

nature

7 August 1980

Towards a more competent Civil Service

The narrow issue of how much scientists working in the British Civil Service should be paid during 1980 will no doubt be settled by this week's arbitration hearing, to which the Civil Service Department has been driven by the Institution of Professional Civil Servants (see *Nature*, 24 July). The outcome, no doubt, will be a compromise between the government's offer and the claim by the IPCS — and a further setback for the system of "pay research" by which salaries are determined. The more serious questions of how, and on what terms, scientists should be recruited to the Civil Service will however remain unasked. As a result, scientists in the public service will continue to be treated as a group apart while the public service and thus the British government will continue to be less effective than it could and should be. It is a scandal that so little attention has been paid, since the publication in 1968 of the report of the Fulton Commission on the organization of the Civil Service, to these important issues.

Traditionally, as the whole world knows, the British Civil Service has been a happy and largely successful hunting ground for gifted generalists, able to turn their attention from the administration of bits and pieces of the British Empire to the administration of, say, the publicly supported schools. It is customary to suppose that in the nineteenth century the system worked splendidly, although with hindsight it is clear that for every brilliant administrator of India thrown up by the system there was a small army of dull pecksniffs guiding the growth of the public service in unimaginative directions. How could it be otherwise, given the traditional method of recruitment into the Administrative Civil Service? Although in the twentieth century the Administrative Class has replenished its ranks with graduates from well connected universities, Oxford and Cambridge in particular, rather than from the sons of well connected families (as in the nineteenth century), the principle is unchanged. It is to take a man (or now, mercifully, a woman) of high intelligence but without particular expertise, and to let him learn the craft of public service on the job in one ministry after another, with spells of duty rarely spanning more than three years. Within the service, frequency of movement is a mark of success, with the result that the high fliers, as they are called, begin and end as generalists — intelligent, to be sure, but almost proud of having no expert knowledge of the fields of the public service they administer. Recognizing, however, that the public service increasingly requires expert knowledge in many different fields, the Administrative Civil Service has built up separate cadres of specialists — scientists and engineers but also economists and architects, for example.

The Fulton Commission was born of a suspicion that this system was increasingly an anachronism. The suspicion was confirmed by the commission's analysis. Its conclusion that generalists, sometimes rudely but properly called amateurs, had outlived their usefulness to society as public servants was understandably unpalatable to those primarily concerned. In the circumstances, it is something to be grateful for that the least radical of the Fulton Commission's recommendations have indeed been followed. The Administrative Civil Service now looks less eagerly to the products of Oxbridge courses in classics or history for its new recruits. Responsibility for recruitment has been transferred from the Treasury to the newly created Civil Service Department, while steps have been taken, as Fulton asked, to make it easier for people with experience of other walks of life to join the Civil Service in mid-career. (Even so, the flux of people into and out of the public service is not nearly as great as Fulton hoped.) Under these arrangements, there is of course no

reason why scientists or people with other specialist backgrounds should not compete for places in the Administrative Civil Service alongside those with other kinds of degrees. Understandably, the numbers of those prepared to ape the generalists at such an early stage in their careers as scientists is small and likely to remain so.

The Fulton Commission acknowledged that this was likely to be the case, for which reason (among others) it urged that there should be a merger of the Science and Administrative classes. Since 1968, British governments have stolidly declined to follow this proposal. Administration, they say, no doubt on the advice of the administrators, is in itself a special skill, distinct from other kinds of skills. The one concession to the Fulton view to have been made in the past decade is a scheme by means of which scientist within the public service can apply for a transfer to the Administrative Branch. Again, understandably, the volume of transfers is a trickle. More seriously, the habits of the public administration are unchanged — those who enter the Administrative Civil Service from some other part of the public service do so on terms spelled out by the administrators.

The result, increasingly apparent, is that the British government is denied the kind of advice and administration that circumstances now demand. The record of simple administrative errors stemming from innocence of technical matters grows with the passage of the months. It is not so long since the renegotiation by the Home Office of the international allocation of broadcasting frequencies robbed the BBC of facilities now plainly essential to its social function. More recently, the British government has been allowed to dither about the payment of the second promised tranche of £25 million to the semiconductor company INMOS (*Nature*, 5 June), apparently in ignorance of the nature of technical development in novel fields. (In the end, as the Prime Minister announced last week, the National Enterprise Board has been allowed to meet its moral obligation.) Unhappily, such conspicuous errors are almost by definition relatively minor. The influence of an ill-informed administration on the evolution of more substantial policies is harder to pin down. The chopping and changing in British energy policy over the past two decades, however, clearly carries the marks of technical innocence. So does the apparent assumption by several government departments that funds for research and development, or education, can be turned off and on as water from a tap. The truth is that, in such fields, uncertainty is more damaging than mere shortage of funds. But elsewhere, again apparently from lack of technical knowledge, the Civil Service conspires with the government to attempt to make water run uphill. How else is it possible to explain the devotion of successive governments (but not the present) to regional policies intended to share such prosperity as the country continues to enjoy more equitably between different parts of the country? The objective, of course, is admirable, but the results have been at best meagre, as economists have been saying all along.

It would of course be wrong to think that maladministration of this kind would vanish if more of the administration of the government's affairs were shouldered by scientists and other specialists. Scientists and other specialists do not have a monopoly of wisdom, and there is also something in the administrators' claim that good judgement in public administration comes with experience. This, however, is merely an argument in favour of the Fulton Commission's proposal that there should be a thorough merging of the scientific and the administrative Civil Services. While many scientists within the public service would no doubt wish to follow the public interest and their own in the pursuit of research and development, others would be able to contribute more effectively than is at present



possible to the routine work of the public service, preventing the howlers which are at present perpetrated by the generalists and helping also to ensure that major issues of public policy are dealt with in a manner that is technically sound and, with luck, imaginative as well. One unconvenanted benefit would be that the

hosts of advisory committees which at present litter Whitehall could be reduced in number — one of the present government's ambitions. Perhaps the first British Prime Minister who happens to be a scientist might take up the shamefully neglected cause of making the British Civil Service more competent.

Radiation standards not yet for changing

The chequered history (see page 550) of the report on low-level ionizing radiation, published last week by the US National Academy of Sciences, should not detract from its value. The report was commissioned by the Environmental Protection Agency and has been prepared by the academy's Committee on the Biological Effects of Ionizing Radiation. It is popularly known as BEIR III to distinguish it from an earlier report in 1972 and from the first draft of this document (released just over a year ago before all the members of the committee had had their say). The issues with which it deals are of great importance, not merely for regulatory agencies such as the Environmental Protection Agency, for those concerned with industrial health and safety (not only in the nuclear industry) but also for the public at large, rightly concerned that there should be a judicious assessment of the potential hazards of ionizing radiation, especially that produced by the nuclear industry. The public importance of the topic has of course been keenly appreciated for the past quarter of a century, with the result that attempts to calculate the hazards of radiation have been made by national governments, independent organizations such as national academies and even by the United Nations. The particular value of BEIR III stems from that feature for which it is likely to be most criticized — for by including the opinions of members of the committee dissenting on scientific grounds from the main report, it serves to dramatize the uncertainties that persist in this difficult field.

The abiding difficulty in the estimation of the consequences of radiation exposure is that of extrapolating downwards, from the often tragic experience of people who happened to be living in Hiroshima and Nagasaki in 1945, those who were treated with X rays for a variety of diseases such as rheumatoid arthritis or ankylosing spondylitis in the 1930s or those who worked as radiographers before the hazards of radiation were fully appreciated, to the rest of us, whose organs receive 100 mrem a year (in round numbers) from natural and unnatural causes — cosmic rays, natural radioactivity such as potassium-40, visits to the orthopaedics people, airline travel and the like. Hitherto, most serious studies of how the extrapolation should be carried out have been based on the assumption that the risk of damage is proportional to the dose of radiation. (The days have long since gone when it was permissible to talk of a "threshold" dose below which radiation entailed no risk of damage.) BEIR III breaks new ground by flirting seriously with the notion that the relationship between the risk of damage and the dose of radiation that produces it may not be linear after all, but rather quadratic, at least where the induction of some kinds of cancers is concerned. (On the genetic consequences of radiation exposure, BEIR III follows earlier studies in affirming that only the linear extrapolation is prudent in the present state of knowledge or, rather, ignorance.) This is why the report has engendered controversy among its authors even before publication.

Superficially, it is true, even to entertain the possibility that the rate of induction of some kind of cancer may be proportional not to D (where D is dose) but to $\alpha + \beta D + \gamma D^2$ (where α is the spontaneous rate, and β and γ are constants to be adjusted to fit the data) looks like a way of resurrecting the threshold hypothesis. For if γ is positive, extrapolation downwards from large to small D will suggest that the risk of harm by small doses of radiation, such as those attributable to krypton-85 in the atmosphere, for example, will be disproportionately less than those attributable to large doses.

The argument of BEIR III by way of justification is plausible but not conclusive. To their credit, the authors have acknowledged this to be the case. The conjecture that to cause a cancer it may be necessary to break the same DNA molecule at two places (not just one) before natural repair mechanisms have had time to do their work is modestly advanced, with as full a list of the objections as anybody could ask for. The experimental data bearing on this point which are marshalled by the report are impressive, but derive mostly from animal studies. The document acknowledges that the information available about the causation of human cancer is not "robust" enough to distinguish between the possible dose-response models, although there is some reason to think that skin cancer does not turn up at low doses of radiation, that the relationship between leukaemia induction and radiation dose among the Japanese bomb survivors is adequately represented by a quadratic relationship but that the risk of breast cancer is linearly related to the dose of radiation.

Inevitably, a quadratic rather than a linear relationship implies that the risk of cancer induction after exposure to small doses of radiation is reduced. BEIR III includes comparisons of the calculated risks based on the two assumptions which suggest that the risk of cancer induction after exposure to, say, 10 rads of ionizing radiation will be halved if the relationship between risk and dose is quadratic, not linear. Superficially, that represents a substantial change, for which reason it is relevant that the earlier calculations (like those of BEIR III) are accompanied by large (and unavoidable) errors; the new estimates, based on the quadratic assumption, do not lie all that far outside the errors of earlier calculations. A more serious difficulty (to which one of the dissenters, Professor Edward Radford, draws attention) is the element of arbitrariness in the way in which BEIR III had to calculate the parameters in its quadratic relationship between risk and dose from a few clumps of data relating to high acute exposures.

How, then, should the regulatory agencies respond to BEIR III? Some, no doubt, will be tempted to say that present regulations for occupational exposure to radiation might safely be relaxed, although not by very much. It will be a mistake, however, if BEIR III is taken as a licence for such a step. For the report itself is properly so hedged about with qualifications (and packed with interesting but complicated evidence to suggest how sensitivity to radiation may vary with age, sex and genetic constitution) that it cannot be regarded as the last word on the subject. Accordingly, the regulatory agencies should for the time being stick to the assumptions on which their calculations have hitherto been based while setting out more energetically to test the interesting proposal put forward by BEIR III. Perhaps the most conspicuous omission from the report is the committee's own assessment of the work that needs to be done more adequately to underpin its argument and conclusion. It is, however, plain that much can be done to test the conclusions of BEIR III. Animal experiments designed to test the risk of specific kinds of tumours after exposure to radiation could help to distinguish between the linear and quadratic hypotheses. BEIR III itself makes much of the results of the classic epidemiological study by Court-Brown and Doll on the incidence of tumours of people treated with X rays for ankylosing spondylitis. It is too much to ask that the regulatory agencies and the nuclear industries throughout the world should apply the same care to the study of the health of the only substantial group now exposed to relatively large doses of radiation — those occupationally exposed in nuclear plants?

Congress turns sour on technology fund

Washington

As part of a general effort to cut the foreign aid budget, Congress is threatening to veto the major US contribution to the new international fund for science and technology in developing countries which was set up following last year's United Nations Conference on Science and Technology for Development (UNCSTD).

The House of Representatives Appropriations Committee last week recommended the elimination from the 1981 budget request of a \$10 million contribution which the Carter administration had proposed for the new fund. The committee also proposed changing the foreign aid bill so as to prevent any of the \$130 million allocated to the United Nations Development Programme — the agency handling the fund — being used for this purpose.

UNDP officials hope that these recommendations will be rejected when the full House debates the foreign aid bill. Otherwise they fear that withdrawal of the US contribution could put the whole fund in jeopardy. This, they warn, would have repercussions for relationships between the US and the many countries — particularly in Africa — which have already submitted project proposals to the fund on the understanding that adequate support has been promised.

Delegates to the UNCSTD conference in Vienna last August agreed to the fund as an interim measure. Initially the developing countries had proposed a more ambitious scheme for a \$2,000 million to \$4,000 million annual tax on technology transfers from the developed nations, but this was quickly rejected as unrealistic.

Promises were, however, made to explore the long-term prospects for such a scheme. Meanwhile a two-year interim fund was proposed under the auspices of the UNDP, with an initial target set at \$250 million, of which the US indicated it would be prepared to cover 20 to 25 per cent.

In practice, even this target turned out optimistic. At a meeting in New York in March, total pledges of \$36 million were made, primarily by Austria, Denmark, Norway, Sweden, The Netherlands and the US. (The UK made it clear in Vienna that it was not prepared to contribute: France and Germany are still negotiating conditions under which they may give up to \$9 million more.)

Even though the total was less than had been hoped for, the UNDP decided that enough had been raised to get the fund off the ground. With the support of the UN General Assembly, the fund officially became operational in May. UNDP has already received over 550 applications from the governments of developing countries, and 70 projects have been selected as candidates for funding.

One African country has approached the

UNDP for joint funding of a new metallurgical research institute, and another eight are already including possible support from the UNDP in schemes to integrate science and technology policies into their five-year economic plans.

UNDP officials argue that for the US to withdraw its proposed contribution — given its central role in engineering the Vienna compromise and steering attention away from the more far-reaching third world demands — would not only prejudice the future of the fund itself, but would also cast a cloud over all future negotiations on overseas aid.

The proposed \$10 million US contribution has already been endorsed by the science committees and the foreign affairs committees in both the House and the Senate. Indeed some congressmen say they would have been prepared the support the \$15 million contribution which President Carter had proposed in his original January request.

Despite this support, however, the appropriations committees are proving harder to convince. Commenting on its proposal that the US should not contribute to the fund, the House Appropriations Committee says that it took the action because the Administration had failed to provide adequate information on the activities of the fund.

This information is now being hastily provided, in the hope that at least some members of the Appropriations Committee can be persuaded to change their position. But the UNDP contribution has also become tied up with the conflict between the Congress and the Administration over the latter's plan to set up an Institute for Scientific and Technical Cooperation (ISTC) as a focus for research relevant to developing countries.

Last year Congress accepted the ISTC proposal in principle, but the appropriations committees failed to allocate funds after a bitter fight over the form that the institute should take, and its relationship to existing scientific and technological programmes in the Agency for International Development.

This year the Administration has again put forward the ISTC for funding. But despite plans to reduce its scope it seems increasingly unlikely that the institute will get off the ground.

Such a failure, embittered as it would be by the disputes which there have been between the Administration and Congress about overseas aid and the growing hostility of Congress towards multilateral aid programmes, imply that supporters of the UNDP have an uphill task ahead of them.

David Dickson

Compromise for Finniston?

On the principle that ministers cannot go on holiday until parliament has seen that their desks are clear, the British government was earlier this week preparing its much-delayed response to the report of the Finniston Committee on engineering education. The response will lose some friends and make some enemies, but is otherwise likely to be a compromise.

The central issue to have become apparent since the committee's report was published earlier this year is whether responsibility for the education of professional engineers, including the accreditation of university and other courses, the definition of professional standards and the certification of those claiming them, should rest with a new public body to be called the British Engineering Authority.

The Finniston Committee argued that there should be such a body, which would inevitably take over from the separate engineering institutions many of their existing educational and professional responsibilities. The committee also asked that such a body should be statutory, responsible to the minister responsible for industry — for the time being Sir Keith Joseph, Secretary of State for Industry. Only by such a device, the committee argued, could the reorganization of engineering education and qualification be

carried out in such a way that industry could take notice. The committee also recognized that even the British government has, on paper at least, a long purse.

Since the publication of the report in February, the Department of Industry has invited comments from no fewer than 400 interested parties, most of whom have apparently replied. Many respondents, the Confederation of British Industries and the Committee of Vice-Chancellors and Principals for example, have however disappointed members of the Finniston Committee by warning the government against the notion that the engineering profession should be regulated directly by the government. This advice is likely to be the more influential because those who offer it also welcome structural changes in engineering education and qualification.

The favourite compromise seems to be that there should indeed be a new authority responsible for engineering education, but that it should be set up as an autonomous institution by the Privy Council, not by the government as such. To begin with, there should be a measure of direct financial help from the British government, but in the long run it might be appropriate that support should be channelled through bodies such as the University Grants Committee.

Radioactive waste

Windscale leak

The scathing report last week from the British Nuclear Inspectorate on the latest radioactive leak from the Windscale reprocessing plant raises familiar issues. Yet again, it seems, a substantial leakage of radioactivity, 100,000 curies this time, has arisen from relatively minor design faults and sheer human frailty.

One of the benefits of the arrangement under which the Nuclear Installations Inspectorate works directly to the Health and Safety Executive is, however, that its full report (Health and Safety Executive, £1.25) has now been made public. It emerges that the inspectors had seriously considered prosecuting British Nuclear Fuels Limited, or members of the senior staff at Windscale, for breaches of the operating licence. Instead, the inspectors have asked the company (which is state-owned) to take remedial action and plainly hoped that publication of their report will be a more effective safeguard for the future. BNFL has duly eaten humble pie, with a statement reading like the public confessions common in China during the Cultural Revolution.

The chronology of this latest incident at Windscale begins in 1955 when a plant was built for bleeding off small quantities of highly active liquid waste from the reprocessing line as part of the research programme then under way at Harwell to classify highly active waste. The UK Atomic Energy Authority (then the operator of Windscale) erected building 701 which contained a 1,400-litre stainless steel tank in which active waste could be aged for an appropriate length of time, and a device for transferring some of this material into 20-litre shielded flasks for transport to Harwell. There were arrangements for returning waste from the building to the main processing line, principally from a small 70-litre sump in the floor of the building.

After the last consignment of waste to Harwell in 1958, Building 701 seems to have fallen out of use and out of mind. For some years, workmen at Windscale followed the standard operating procedure of emptying the sump at regular intervals, but during the 1970s the practice had become irregular. BNFL told the inspectors that they assumed that the liquid collecting in the sump was not radioactive, but probably consisted of rainwater leaking through the roof of the building and of condensation inside. The inspectors complain that BNFL should have considered the chance that active waste was splashing over the movable vanes used in a valve arrangement to divert the stream of active waste in one direction or another.

Attempts to empty that sump during the 1970s were not always successful, now it seems because one of the two outlet lines had been mistakenly cut and blanked off in

1971. Curiosity about the level of liquid in the sump could not reliably be satisfied — the pointer on the face of the instrument recording the liquid level can apparently turn full circle and then keep on turning, so an over-full sump may seem nearly empty.

The leak was discovered during a survey of the Windscale site in 1978, and recognized to consist of fission products only two years old in March 1979 when the nuclear inspectors were informed. The inspectors say that for several years the sump had been overflowing and that radioactive material had found its way through the stainless steel cladding lining the bottom of the building to soak through the concrete foundation to the ground beneath.

The penance required from BNFL includes the reinstrumentation of building 701, its isolation and, eventually, its decontamination, the monitoring of radioactivity in the ground beneath the plant (where most of the activity is some three metres beneath the surface) and preparation for the physical isolation of the activity or even the removal of the contaminated soil.

Both parties to the investigation agree that nobody has been exposed to a significant radiation dose because of the leak.

European biotechnology

EEC proposals

The European Commission's proposals for the regulation of genetic manipulation, once widely feared for their restrictiveness, have turned out to be comparatively tame. The commission's "recommendation", submitted to the Council of Ministers this week, requires merely that experiments in genetic manipulation should be registered nationally. The proposals are thus much less constraining than the directive proposed in 1978 and later withdrawn.

A "recommendation" carries no legal force but, if agreed by ministers, has a certain moral force among the nine member states. Last week's recommendation argues that while hazards are now thought to be "non-existent or small" it would nevertheless be prudent to register experiments centrally in case so far undetected "long-term effects" exist. To deal with commercial objections about confidentiality, notification would be restricted to "the portion of the experimental protocol which is required for the evaluation of safety".

Compulsory notification already exists in the United Kingdom, and may do so in Germany if a proposed law on genetic engineering re-emerges after the October election. Elsewhere it is voluntary.

The commission would also set up a committee of experts, mandated by member states, to examine at least once a year the need for harmonization of regulations and to consider emerging knowledge of hazards. **Robert Walgate**

IWC whaling quotas

Politics wins out

The recent 32nd annual meeting of the International Whaling Commission (IWC) closed after a week of political struggle between those nations seeking a ban on commercial whaling (led by France, the Netherlands and the United States) and those, mainly the whaling nations (Canada, Chile, Iceland, Japan, South Korea, Peru, South Africa, Spain and the Soviet Union), arguing that such a ban could not be justified.

Any hope of a total moratorium was crushed at the beginning of the meeting, and for most of the week it looked as if agreement on quotas might never be reached. On the final day of the meeting, however, quotas were eventually agreed — 13,753 whales of all species can be taken in 1981–82 compared with 15,883 in 1980–81. The alternative of no agreement at all might have led to the demise of the whaling commission and a free-for-all, which would have been an even greater blow to the conservationists.

The purpose of the IWC's annual meetings is to reach agreement on the management of whale stocks for the next year based on the advice of its scientific committee. One of the problems is that the monitoring and prediction of whale population dynamics is fraught with difficulties, allowing interested parties to interpret the statistics as they wish.

The task of the IWC's scientific committee, which met a few weeks before the commission's main meeting, is to formulate a basis on which catch quotas can be set. It does this by estimating the current population of individual whale stocks and the ideal population which could be supported in the absence of interference by man. A population slightly below the ideal level will replace itself more rapidly than one at the ideal level because of increased resources per whale. The aim is to maintain populations at the level which shows the greatest replacement rate and hence gives the maximum sustainable yield (MSY). Under the IWC's new management procedure, a target yield, which provides guidance for setting catch quotas, is set at 90 per cent of MSY. The management procedure also stipulates that all catching should stop on any stock which falls to 10 per cent below the level at which it produces the MSY.

Estimated stock sizes and MSY and recommended catch limits

North Pacific, Western Division	Males		Females
	(11+)	(10+)	
1910 stock $\times 10^3$	157.9	175.0	
1981 stock $\times 10^3$	66.5	124.6	
1981/1910	42.1%	71.2%	
MSY level $\times 10^3$	88.3	131.1	
1981% MSY level	75.3	95.0	
Catch limits	0	405	

The ideal population can be estimated only by knowing the current population and its rate of change. Almost all the data on the status of current stocks have come from whaling ships which have been providing information on the basis of catch per unit effort. This works on the principle

outcome of the recent meeting as much as might have been expected. In spite of Beddington and Cooke's new evidence on sperm whales, which led the scientific committee to recommend that they should not be killed in the North Pacific, a quota of 890 was awarded to Japan. Some

IWC whaling quotas

	1980/81	1981/82		1980/81	1981/82
<i>North Atlantic</i>			<i>S. Hemisphere</i>		
Sperm	273	130	Bryde's	264	264
Minke	2,543	2,554	Sperm	580	300
Sei	100	100	Minke	8,102	6,718
Fin	624	701	<i>Others</i>		
<i>North Pacific</i>			Bowhead	18	17
Bryde's	479	529	Humpback	10	10
Sperm	1,350	890			
Minke	1,361	1,361			
Gray	179	179	Totals	15,883	13,753

that the difficulty of finding and catching a whale is inversely proportional to the total number of whales available. Changes from year to year in making a catch will reflect change in the population size and hence allow estimates of ideal population to be made.

Catch per unit effort, however, is not a reliable measurement on which to base calculations of population because it varies considerably with different whaling techniques. When allowances for handling time were recently taken into account, it was found that the depletion of population was being seriously underestimated.

One new way of modelling populations which overcomes some of the difficulties with catch per unit effort was proposed by John Beddington and J G Cooke of the University of York at the recent scientific committee meeting. It has already been applied to sperm whale stocks in the North Pacific with some success. The Beddington and Cooke model uses the fact that whaling distorts the distribution of length in populations as the basis for calculations of current and ideal populations (whaling ships tend to catch big animals leaving smaller ones behind). By examining the current length distribution of the catch and the change over recent years and by knowing the size of the catch, the current population and hence the ideal population can be calculated. This technique works well for species of whale, such as the sperm whale, which vary considerably in length.

One difficulty with the management procedure is that it only works well for species which have already been depleted where the catch is a fair proportion of the current population. The minke whale, for example, the most heavily exploited species, cannot be managed easily because its numbers are actually on the increase. Sperm whales also present problems, because unlike all other species, they are polygamous. Only large males over 25 years of age can hold harems so the damage to the sperm whale population of taking only the largest whales could be greater than at first suspected.

The deliberations of the scientific committee probably did not affect the

concession to the scientific evidence was made, however, by banning the killing of female sperm whales and all males over 45 feet long. Two other seriously endangered species also failed to get total protection: the humpback whale of the North Atlantic — Eskimoes in Greenland will be able to kill 10 next year — and the bowhead whale in the Bering sea where a small quota has been set for the benefit of Eskimoes in Alaska.

Judy Redfearn

More Soviet psychiatry

Physicist held

Vladimir Kislik, nuclear physicist, long-term refusenik and member of the European Physical Society, has been committed to a Kiev mental ward, apparently to prevent him from meeting western visitors to the city. Although this means of repression has of recent years been used against a number of political dissidents, including geneticist Zhores Medvedev and mathematician Leonid Plyushch, this is the first time that it has been used against a Soviet scientist who simply wishes to emigrate to Israel.

Kislik applied for an exit visa in 1972, but was refused on the grounds of access to state secrets while at his previous job at the Chelyabinsk Nuclear Reactor Plant. Following his application, he was dismissed from his research post at the Institute of Physics of the Ukrainian Academy of Sciences and since then has had to work as a bus-ticket seller, nightwatchman and the like. He attempted, however, to keep up his scientific reading and to organize a scientific seminar for other refuseniks in his own home. In June 1976, however, he was beaten up by security police and threatened that he must close his seminar — or else face a criminal charge of black market activities. Meanwhile his name "disappeared" from the Soviet bibliographies and from references to the papers which he had published in his professional capacity.

He did, however, have one unpublished paper on the "Absorption of Helium by

Irradiated Samples of Austenitic Steels". Before his dismissal from the institute, this research had been cleared for open publication. Kislik managed to send the paper by hand to the United States, where in 1977 it appeared in the *Journal of Nuclear Materials*.

Shortly afterwards, an article appeared in the local evening paper *Vechernyi Kiev*, accusing Kislik of being a foreign agent and of smuggling out nuclear secrets. At the end of 1979, the accusation was revived, and he was informed by the Kiev Public Prosecutor, Ignatiev, that a file had been opened against him under Article 136 of the Ukrainian Penal Code "Violation of Authors' and Inventors' Rights" — in other words, plagiarism.

Since October 1979, Jewish emigration from Kiev has virtually ceased and routine police harassment of long-term refuseniks has intensified. In April 1980, Kislik was prevented from travelling to Moscow for the refuseniks' International Symposium on Collective Phenomena. One of the western visitors to the symposium, Professor Paul Kessler of the Collège de France, later



visited Kislik in Kiev. After the visit, Professor Kessler reports, he was attacked by four strangers in plain clothes who gave him an "intentionally not too heavy" beating, which, he believes, was meant simply to intimidate him. A few weeks later, Kislik managed to make contact with some American geneticists who were attending a meeting in Kiev — an event which, some of his friends believe, was not unconnected with the latest harassment.

On 25 June, Kislik was picked up in the street and taken to the police station for an identity parade in connection with an alleged theft. On 4 July, he was arrested for alleged hooliganism and given 15 days detention. When this term was up, and he was not released, he threatened to go on hunger strike. The next day, 19 July, he was transferred to a psychiatric ward in the Pavlov hospital. Ironically, and to a certain extent in support of Soviet claims that Jews (as opposed to "Zionists") suffer no discrimination in the Soviet Union, the doctor in charge of the case is a certain Naum Lifshits.

Vera Rich

Neuroscience

Snyder's pad

Washington

The Johns Hopkins Medical School in Baltimore has joined the growing ranks of United States research universities in announcing its intention to set up a new department devoted to neuroscience. This will be the first new department to have been established at the medical school for twenty years. Under the direction of the new department's chairman, Dr Solomon H. Snyder, who will become the medical school's first professor of neuroscience, its research will be concerned with the chemical and pharmacological aspects of brain function. Dr Snyder, joint winner of the 1978 Albert Lasker Basic Medical Research Award for his work on the actions of neurotransmitters and, in particular, on the functioning of opiate drugs, is this year's president of the American Society of Neurosciences.

The proposal to set up the department was made by an *ad hoc* group appointed by



the medical school last autumn. The committee said that there were "compelling academic grounds" for a new department and pointed out that although knowledge in the field of neuroscience dovetailed with that in other disciplines, there was now a common body of knowledge and methodological approaches shared by people trained and working in neuroscience.

Bringing together the neurological aspects of various traditional disciplines into a single department will have organizational as well as academic advantages. According to Dr Steven Muller, president of Johns Hopkins University, "although Hopkins has universally recognized strength in several areas of neuroscience, the School of Medicine now needs the cohesive focus in neurobiology so necessary for the develop-

ment of exciting and rigorous teaching programmes for medical students and for predoctoral and postdoctoral students in neuroscience".

One of the immediate research interests of the new department will be a study of effects of Huntington's disease on enzyme activity in the brain. This will form part of a nine-project research programme on the disease which last week received a grant of \$1.8 million from the National Institute of Neurological and Communicative Disorders and Stroke.

David Dickson

Byelorussian graduates

Missing out

Young Byelorussian graduates are failing to take up their appointed posts in the Soviet national economy, according to a recent survey by the Byelorussian SSR State Committee for Labour.

According to Soviet practice, all young persons completing higher education must work for a prescribed period (usually three years), in whatever post the relevant ministry assigns to them. These posts can be anywhere in the Soviet Union, not necessarily in their own home republic. In Byelorussia, however, the system seems to have broken down. In 1978, says the survey, over 3,000 newly qualified specialists — just under 5% of the total — failed to turn up at their appointed enterprises.

In all, out of the 120,000 persons holding higher qualifications in agriculture 41,000 are now working in non-agricultural jobs, 10,000 with teaching qualifications are not teaching and 13,000 university and technical college graduates are working in branches of industry, building and transport unrelated to their education. Over 25% of the young specialists who have graduated in the past four years are now doing jobs which require no higher qualifications.

The ministries themselves, it appears, are partly to blame. A spokesman for the Byelorussian State Committee for Labour explained that many ministries distribute their young graduates at random, often switching them from the job for which they were originally destined letting them go off and find work for themselves, or terminating their assigned job earlier than scheduled. An increasing number of departments are refusing to employ graduates along the lines for which they have been trained. Cases of patronage and "protection" are also said to account for an unspecified number of graduates failing to fulfil their "civil obligations" of directed work.

Nor surprisingly, the professional skill of management workers in Byelorussian industry and agriculture is also said to be "rather low" and causing "great concern."

Vera Rich

Radiation risks

Twice lucky?

Washington

After a year of intense internal debate, the US National Academy of Science has issued a report revising its earlier estimates of the health risks of low-level radiation, and indicating that these may be lower than it had previously suggested.

In particular, academy scientists have qualified their support for the linear "no threshold" model for predicting dose-response relationships at low levels of radiation. They now say that, given all the uncertainties involved, a linear quadratic relationship is more likely to be realistic and that the linear hypothesis should be considered merely to define the upper limits of risk.

The report, which was prepared by the Academy's Committee on the Biological Effects of Ionizing Radiation (the BEIR Committee) and was commissioned by the Environmental Protection Agency, does not recommend specific exposure levels. But its findings are likely to reduce pressure on the agency to develop more stringent regulations.

The committee's conclusions, however, are still under fire. The chairman of the subcommittee on somatic effects, Dr Edward Radford, claims that support for the linear hypothesis was downgraded on the basis of a biased selection of data, giving too much weight to laboratory studies and not enough to epidemiological evidence. Another committee member, Dr Harald Rossi claims that the committee has not gone far enough in its revisions, and that the dangers are still being overstated.

The BEIR Committee first gave its support to the linear hypothesis in its initial report, published in 1972. Given the many uncertainties surrounding attempts to estimate the effects of low-level radiation, and in particular the impossibility of providing conclusive statistical evidence based solely on epidemiological studies, the committee proposed the linear hypothesis as a basis for regulation.

Since then, it has been used as the basis for standard setting by the EPA. But it has been challenged by the nuclear industry, which claims that it provides an unrealistic assessment of the health risks of nuclear power, and thus could lead to overly-restrictive legislation.

The BEIR committee came out with a revised version of its report last spring. It again endorsed the linear dose-response relationship as a basis for regulation and provided estimates of the several hundred additional cancer deaths per million population that would result from continuous exposure to one rad of radiation.

Publication of the report, however, brought into the open a fierce debate which had until then taken place within the committee over how much weight to give to

different types of evidence — and the appropriate dose-response models which could therefore be justified.

Five members of the committee issued a dissenting statement, claiming that insufficient weight had been given to radiobiological data and that accepting the linear hypothesis as a basis for regulation, would result in regulations which would hinder the development of nuclear energy and the use of radiation in medicine.

When it became clear that additional members of the committee also had reservations, academy president Dr Philip Handler decided that the controversial section should be rewritten. The revised report was published in Washington last week, with dissenting statements from Drs Radford and Rossi.

The new report goes some way to establishing a compromise on what the

academy calls the “ideological differences” between committee members over methods for estimating numerical risk values for cancer induction.

Whereas previously, for example, the committee had used data on general cancer incidence taken from survivors of the Nagasaki atomic bomb explosion, the new report gives this data less prominence than the cancer mortality data on survivors in both Hiroshima and Nagasaki, which suggests a more quadratic dose-response relationship at low radiation levels.

Similarly, rather than giving estimates of predicted increased cancer mortality and morbidity based directly on the linear hypothesis for the effects of low LET (linear energy transfer) radiation — primarily X-rays and gamma rays — the new report presents a range of risk estimates based on linear, linear-quadratic

and quadratic models. And it adds that the linear-quadratic is “most consistent with existing human epidemiological studies and radio-biological data and knowledge”.

Dr Radford took no part in the rewriting process. He remains critical of the final results, claiming that the report ignores much new data in support of the linear hypothesis, with the consequent risk of understating the dangers of radiation.

Dr Rossi, too, remains dissatisfied. He claims that his own work and that of others on the effects of radiation on plant cells suggests that the linear hypothesis overstates the dangers. And that even though its prominence has been downplayed, it is still likely to be used in standard setting, to the disadvantage of the nuclear industry.

David Dickson

Calculations and measurements

What follows is a summary of the definitions in BEIR III relating to the somatic effects of ionizing radiation. The terms of reference of the study, set by the US Environmental Protection Agency, include an explicit reference to nonlinear effects. The report therefore includes a discussion of the small-scale effects of the interaction of radiation with matter along the following lines.

Radiation dose (D) is a quantity of energy deposited in matter; the unit is the rad (equivalent to 100 ergs of absorbed energy per gramme), soon to be replaced by the SI equivalent of the gray (or Gy), with 1 Gy = 100 rad. Charged particles (e.g. α particles) or uncharged and indirectly ionizing particles (e.g. X rays) differ from each other in the rate of “linear energy transfer” (LET) along their tracks; for a given particle, the LET is also a function of energy. The LET is low (a few keV per μm) for cosmic-ray μ -mesons, high for α -particles.

Concerned as it is with nonlinear effects, BEIR III needs to introduce a microscopic analogue of the macroscopic dose D called “specific energy” z and intended as a measure of energy deposition by radiation in sub-cellular structures such as cell nuclei.

The deposition of energy along the track of a particle is not uniform on such a scale, because the interactions of radiation with matter entail interactions with discrete entities in the irradiated matter. Thus although (with appropriate units) the average value of z (written $\bar{z}$) must equal D , the microscopic intensity of energy deposition can vary dramatically with different kinds of radiation.

The report goes on to consider (but does not eventually adopt as an assumption) the proposition that for radiation to cause somatic damage, two “sublesions” must occur within some small range (say 1 μm) in a cell and within some specified interval of time. Then the frequency of “lesions” (E)

will be $K(\bar{z}^2)$ where K is a constant. Moreover, the mean square microscopic dose will be related to the macroscopic dose (D) by $\bar{z}^2 = wD + D^2$ where w is a constant determined unambiguously by the statistical properties of z for the type of radiation concerned.

One consequence of this argument is that even the assumption that it takes two sublesions (“hits”) within some microscopic region leads to a dose-effect relationship in which the risk of damage is of the form $\beta D + \gamma D^2$, but in that case β and γ should be related to each other by the statistical properties of the radiation concerned, whence the relative biological effectiveness of high-LET and low-LET radiation should be calculable.

With this said, BEIR III says that wherever possible empirical data have been fitted to curves with the form $(a_0 + \alpha_1 D + \alpha_2 D^2) \exp(-\beta_1 D - \beta_2 D^2)$ where all the constants are positive, a_0 represents the spontaneous rate of some effect and the exponential term (significant only at high doses) takes account of competition from cell death.

BEIR III includes a discussion of using human epidemiological data as a means of determining dose-response relationships empirically. Apart from familiar problems of bias (people exposed to diagnostic X-rays may be especially prone to cancer, for example), the report draws attention to the retrospective character of most epidemiological studies, the problems occasioned by the long latency period for cancer induction, the distinction between mortality and morbidity (and the possibility that the latter may be under-reported), differences of susceptibility between different genetic groups.

Numerical calculations of cancer caused by radiation yields rates per million people assumed to be taken from a population represented by life-tables for the United States population in the period 1969–71.

The BEIR report uses two alternative models for calculating the “excess” effects of radiation. Absolute risks from radiation exposure are represented by constant probabilities of cancer formation, or of death therefrom, for each year of life after the lapse of the latency period. Relative risks are calculated on the assumption that the effect of radiation exposure is to increase the formation of cancer, or of cancer mortality, in proportion to the spontaneous rate at the age at which the damage becomes apparent.

Like previous analyses of the consequences of radiation exposure, BEIR III leans heavily on data gathered over the years from the populations of Hiroshima and Nagasaki, but again it breaks new ground by seeking to separate the effects of gamma and neutron doses at the two explosions. The two explosions differed in that at Nagasaki the explosion exposed the population to much smaller neutron doses than that at Hiroshima.

To make the calculations of risk explicit, BEIR III considers three separate cases of radiation exposure: (a) a single exposure of a population represented by the US life-tables to 10 rad of low-LET radiation; (b) a continuous lifetime exposure at a rate of 1 rad per year; and (c) a pattern of exposure to 1 rad a year at ages chosen so as to simulate occupational exposure to radiation.

Different assumptions give very different results. Spontaneous cancer mortality for the standard US population is calculated at 16.38 per cent, and the consequences of a single exposure of the whole population to a dose of 10 rad turns out to be an extra 766 deaths (0.47 per cent) on the absolute risk calculation and an extra 2,255 deaths (1.4 per cent) on the relative risk basis, on the assumption that the dose-effect relationship is represented by a quadratic function including a linear term.

BEIR III is so far distributed in typescript form, but will be available in book form from the National Academy of Sciences later in 1980.

Alternative energy

Europe stirs

In Britain and France, there are now signs that governments are prepared to back alternative energy sources with real money. Last week, M Andre Giraud, the French industry minister, announced a 50 per cent increase of the solar energy budget. Hard on his heels, the UK government will announce this week a plan to spend £6.5 million on geothermal and wind energy projects.

With the latest increase, the French solar energy budget will amount to an annual £21 million. The *Commissariat à l'énergie solaire* (Comes) estimates that France may produce 7.5 to 9 million tonnes of oil equivalent (mtoe) from biomass by 1990, more than 10 million mtoe by 2000 and ultimately 30 mtoe.

In 1990, solar heating is expected to provide 1.3 to 1.5 mtoe, geothermal energy 0.8 to 1 mtoe, and "microcentrales" (small solar power stations) 0.4 to 0.5 mtoe. Together with biomass, these sources will furnish 5 per cent of French energy needs compared with 1 per cent now.

Biomass is the centre of the French programme and forest wastes, used for methanol production and rural energy needs, its chief element. There is also a plan to use agricultural wastes and, in the process, to develop fermenters and distilleries which can be exported to Third World countries. There are somewhat pious hopes that these countries, using local biomass, would then re-export liquid and gaseous energy products to France, thus ultimately reducing French dependence on OPEC countries.

In Comes's vision, French agriculture would also be transformed, with more acreage devoted to energy crops and some 60,000 to 80,000 jobs created.

In Britain, the Department of Energy has recently estimated that some 8 per cent of Britain's energy requirements could come from biomass (including 2-3 per cent from burning domestic rubbish), while only two years ago the estimates were about 0.5 per cent. These estimates are however arrived at differently from the French, and represent a large number of small contributions from the energy-efficient use of farm and industrial waste, and finding sites for energy crops.

The major UK commitment announced by the Department of Energy this week is to geothermal "hot rocks" by means of a £7.2 million, three-year research programme at the Camborne School of Mines in Cornwall. The United Kingdom will provide £6 million and the European Commission £1.3 million for the project, which is intended to demonstrate the feasibility of a 30 MW (thermal) generator involving paired wells, 6,500 ft deep, and 2 million m² of fractured rock surface. The project is "complementary" to that at Los Alamos in the United States,

using explosive fracturing rather than high water pressure to create the surface, project leader Dr A W Batchelor said this week. The entire land area of the United Kingdom could be exploited for geothermal energy production, said Batchelor, producing electrical energy at 10 per cent efficiency, if commercial drillings could be constructed to reach 7,000 to 8,000 metres depth. (Current deepest North Sea wells are 6,000 metres.)

A further grant of £450,000 goes to Dr Peter Musgrove of the University of Reading to develop a 25-metre diameter, 130 kW vertical axis windmill, the prototype of a 2-3 MW device. The North of Scotland Hydroelectric Board is showing great interest in wind energy, which is increasingly seen as the most advanced of the alternative sources in the United Kingdom.

In Germany, a question in the Bundestag last week led to the publication of figures which showed a decrease in the non-nuclear energy research programme for the first time since 1972. By 1979, spending in the programme had reached £164 million but it fell to £148 million this year. Within the decrease however, — alternative energy research managed to score a rise from £20 million (1979) to £25 million (1980). This year's budget includes £14 million on solar, £6 million on wind and £4 million on geothermal energy. Comparable figures for the Department of Energy in the United Kingdom are £1.7 million on solar, £1 million on wind and £2.1 million on geothermal.

Robert Walgate

Alternative technology

Water play

Villagers from two very different developing countries, Kenya and Nepal, are to benefit from the results of a project to improve the efficiency of watermills used to grind corn. The project has been stimulated by Jean Gimpel, a European historian of science and technology, who says that several international agencies are taking an interest.

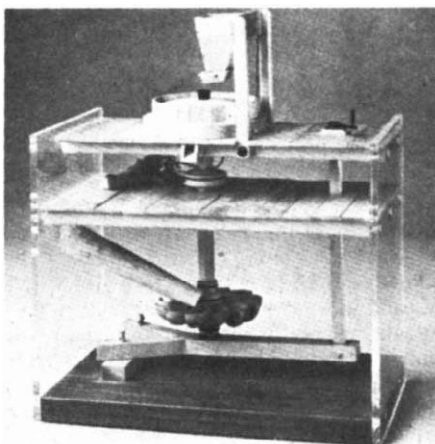
Vertical axis watermills appear to be in common use in hilly regions of many developing countries (and can even be found in some industrialized countries). They have traditionally been used to grind corn for small communities of farmers. M. Gimpel, whose cause is the revival of traditional technologies in rural areas, first interested the Nepalese in modernizing traditional watermills by taking to Nepal a model of a watermill of improved design. Last year, the Research Centre for Applied Science and Technology (RECAST) at the University of Kirtipur set up a project to build a prototype mill in the Kathmandu valley under the supervision of Mr C. B.

Joshi. M. Gimpel says that the Agricultural Development Bank of Nepal has been sufficiently impressed with the project to have distributed models of the prototype to all its branches. The bank has also set up a loan scheme for millers who want to improve their own mills.

The RECAST watermill succeeds chiefly by using well designed bucket-shaped vanes which can accept the full impulse of the water. Replacing the pin mounting of the turbine shaft with ball bearings also increases efficiency, as does enclosing the water feed to the mill in a covered chute. Gimpel and Joshi say that all the work can be done with local skills, mainly the carpenter's who can carve new paddles to the specified design.

Mr Joshi has compared the performance of the improved and traditional mills and says that the modified watermill is 80 per cent more efficient. The power output is increased by 81 per cent, the speed by 48 per cent and the productivity by 50 per cent.

The improvements also include a millstone cover to prevent loss of flour during grinding, a system for collecting the flour to save time and wastage in brushing



the flour off the stone and electric light generated by a small dynamo driven by a bicycle wheel.

Although the improvements cost money, Mr Joshi estimates that the extra costs should be repaid more than three times within a year. He also says that the increased power output of the mills could be used to drive other machines — rice dehousers, trip hammers and bellows and circular saws or lathes. He foresees small industries based around the mills, rather similar to the industrial complexes of eighteenth century England.

The Kenyan government, which has taken up the project since seeing models of the Nepalese mill at the United Nations conference on Technical Cooperation between Developing Countries in Nairobi in May, is hoping to have a small complex of several full-scale improved mills operating near Nairobi for next year's international conference on renewable energy.

Judy Redfearn

NEWS AND VIEWS

Pre-Pleistocene climates

from Samuel M. Savin

ATTEMPTS to reconstruct the history of the world's climate and to understand its changes are among the oldest of geological problems. While enormous progress has been made in recent years, especially in understanding Cenozoic (0 to 65 Myr) climates, it is sobering to read Lyell's¹ 1837 summary of paleoclimatology and to realize for how long geologists have been struggling with similar climatic questions. One problem which continues to create difficulties is the host of variables which can be summarized under the term climate (e.g. average and seasonal variation of temperature, average and seasonal distribution of rainfall, aridity, wind, etc.) while only one, or a very few, are manifested in any single type of deposit. Furthermore, many of these climatic variables can only be documented in a qualitative or semi-quantitative fashion.

An exception to this state of affairs is the climate of the past million years or so. A great deal is now known² about the nature of glacial and interglacial stages and the timing of each. It has been possible to develop testable hypotheses of the causes of glacial-interglacial fluctuations and to develop models of glacial-stage atmospheric circulation³.

Further back in time interpretation of climate becomes increasingly difficult. At a recent symposium on Pre-Pleistocene Climates*, J. Imbrie (Brown University) pointed to recent advances in Pleistocene climatology. Two types of models which are likely to prove especially useful are Global Circulation Models (GCM's) and models which can be thought of as sensitivity studies, designed to test the sensitivity of the climate to proposed changes in some controlling variable (e.g. insolation, atmospheric chemistry, continental and oceanic geometry, etc.). GCM's have been applied to the study of atmospheric circulation 18,000 years ago³ with promising results. L. Gates (Oregon State University) discussed the kinds of geologic information needed if GCM's as

well as simplified models are to be applied to the study of Mesozoic and Paleozoic climates.

Geologic information becomes increasingly generalized and climatic models must become correspondingly less detailed as we proceed backward in time. Pre-Jurassic deep sea sediments have been entirely swallowed up in subduction zones so the record of marine climate is found only in those continental shelf sediments that have survived erosion or metamorphism — a scattered record at best. However, so little is known about the climates of Paleozoic and Precambrian times that even a small amount of climatic data may answer key questions. For some parts of geologic history zero-dimensional climatic models, yielding average global temperatures, can be most useful. In earliest geologic time, for example, J. Pollack (NASA-Ames Research Center) noted that almost any acceptable theory of solar energy production yields solar output only 70 or 80 percent of the modern value. The geologic evidence, however, suggests that temperatures during at least much of the early Precambrian were as warm as, or warmer than today's. Any interpretation of the mechanisms controlling Precambrian climate must resolve this dilemma.

Deep sea sediments, when they are available, provide the most readily interpretable climatic information. The two most common approaches to obtaining paleoclimatic information from deep sea sediments are those based on paleontology and those based on oxygen isotope analyses. Haq (Woods Hole Oceanographic Institute) demonstrated his method of identifying acme events, or peak periods of environmentally (including climatologically) induced floral and faunal migration. Identifying such events involves obtaining fossil census data covering a broad geographical area. The unwieldy census data is reduced into a manageable number of factors, the dominance of each of which is presumed to be environmentally controlled. The migration

of the factors through space and time is then interpreted in terms of variations in environmental conditions.

Oxygen isotopes have permitted detailed interpretation of surface and bottom water temperature history throughout the Cenozoic. While there is some debate, especially for the late Cenozoic, as to the extent to which isotopic variations reflect temperature change and the extent to which they reflect the growth and retreat of continental ice, isotopic fluctuations can be correlated remarkably well from place to place and clearly reflect climatic variations. Berger (Scripps Institute of Oceanography) characterized the Cenozoic isotopic record as showing step-like transitions from one state to another and attempted to discriminate between externally forced climatic changes and those generated internally within the earth-ocean-atmosphere system. He suggested, as did Thierstein (Scripps), that a number of events including the terminal Cretaceous extinction event and the rapid growth of Antarctic ice in mid-Miocene time belong to the latter class. Moore and Pisias (University of Rhode Island) examined the Cenozoic isotopic record, concentrating on variability rather than absolute values of temperatures. Using this annual approach they were able to show that the climate is substantially more variable at some times than others. Highly variable climates may turn out to be characteristic of less stable climatic systems, easily shifted from one state to another. The oxygen isotopic compositions of pre-Cretaceous fossils have frequently been altered by diagenetic processes. As pointed out by Savin, and by Hallam (University of Birmingham) the isotope record adds little, if anything, to our understanding of Jurassic climates. Uncertainties in the interpretation of the isotopic data are as large as or larger than uncertainties in non-isotopic estimates of Jurassic temperatures. Perhaps surprisingly, isotopes, especially of cherts, promise to be useful in providing constraints on early Precambrian temperatures. While the uncertainties in the isotopic temperature are indeed large, they are smaller than uncertainties in temperatures estimated by other means.

*The symposium was held on May 22, 1980 in Toronto at the Annual Meeting of the American Geophysical Union and was part of a study by the Geophysics Study Committee of the National Research Council. The aims of the symposium and study are to summarize the status of Pre-Pleistocene climatology and to identify key areas for future research. A final report will be published by the National Academy of Sciences in 1981.

Samuel M. Savin is in the Department of Geological Sciences, Case Western Reserve University, Cleveland.

In assembling the climatic record on the continents it seems difficult to avoid the anecdotal approach. Terrestrial deposits are widely scattered and are subject to local variations in climate. Climatic variables other than temperature are far more important in affecting terrestrial deposits than marine. However, in spite of these difficulties, Wolfe (US Geological Survey) was able to show that Cenozoic climatic changes derived from North American plant fossils paralleled those of the North Atlantic from planktonic foraminifera. Eugster (Johns Hopkins University) showed a climatic reinterpretation of the Eocene Green River evaporites suggesting a climate similar to that of Holocene Southeastern California. Van Houten (Princeton University) assembled data on ancient laterites and calcretes from which he was able to draw consistent conclusions

about climatic zonations. Crowell (University of California, Santa Barbara) assembled and critically evaluated evidence for Paleozoic and Precambrian glaciations. He offered a hypothetical series of plate reconstructions for Precambrian glacial stages not based on geophysical constraints but drawn so as to cluster glaciated regions in polar areas. Uncertainties in the paleomagnetic data are sufficient to permit considerable freedom in choice of continental positions. While lagging behind our knowledge of oceanic climates, knowledge of terrestrial climates is becoming more complete and techniques for obtaining that knowledge are becoming more sophisticated.

The oceans carry a significant amount of heat, and hence changes in oceanic circulation can be expected to have affected climate. Berggren (Woods Hole) presented a correlation between the climate of the past 200 Myr and the opening and closing of oceanic gateways (or barriers to

circulation). At the time of the Eocene-Oligocene boundary there is a correlation between the opening of the Greenland-Norwegian Sea to the Arctic, the breaching of the Rio Grande Rise and the possible initial opening of the Drake Passage on the one hand and the marked cooling of oceanic bottom water and terrestrial climates on the other. He also pointed to a correlation between the elevation of the Isthmus of Panama 3 Myr ago and the onset of Northern Hemisphere continental glaciation.

In a field as complex and wide ranging as the history of climate throughout the history of the earth it is not surprising that the extent of our knowledge so often seems small. However, it was apparent at the symposium that great strides have been made in the past 15 years in pre-Pleistocene paleoclimatology, not only in the acquisition of climatic knowledge but in the development of new tools and techniques.

1. I. S. Yell, C. *Principles of Geology* (1837).
2. CLIMAP Project Members *Science* **191**, 1131 (1976).
3. Gates, W. L. *Science* **191**, 1138 (1976).

Towards a submarine hydrology

from Marcus G. Langseth

THE excitement generated by the discovery of hydrothermal fields at the axes of the Galapagos and the East Pacific rises notwithstanding, seawater movement within the crust also dominates geothermal heat transfer in the older and colder midoceanic ridge flanks. The pore fluids involved in this circulation system are at low temperature (usually less than 50°C at the top of the crust) and flow rates are slow (centimeters per year). If the high temperature hydrothermal zones at ridge axes are analogous to volcanic and geothermal zones such as Iceland, the low temperature circulation on the ridge flanks may be likened to groundwater movement. The phenomena of submarine hydrology may have little intrinsic glamour but their scientific and practical importance is nevertheless great. Several groups, particularly in the United States, are busily exploring ridge-flank thermal regimes and the past two years have seen some interesting and puzzling developments. Many of these emerged at the last three meetings of the American Geological Union.

The evidence that cold bottom-water circulates below the seafloor consists of temperature gradient measurements which show that temperatures at the base of the sedimentary layer are much less than those expected if the crust were in conductive equilibrium with the lithosphere. Anomalously low crustal temperatures are usually found where the sedimentary cover is thin (less than 200 meters) and where there are frequent exposures of the igneous oceanic basement. The inference is that bottom-water enters the igneous crust at

these exposures and moves laterally below the sediments through a basement made permeable by extensive fracturing, thus carrying away much of the geothermal heat. It has been assumed that this circulation is driven by geothermal heat so that it is a kind of channelized convection, but recent observations have brought this simple model into question.

These observations were made on the flanks of the Mid-Indian Ocean Ridge by the Lamont-Doherty group late in 1977. They involve lines of closely-spaced temperature gradient measurements made with a temperature probe two to five meters long which is lowered into the sediments as it is towed over the bottom by a surface ship. Sonic reflection profiles over the same area throw light on the structure of the sediments. The heat-flow variations suggest that there is cellular convection in the crust and that some of the discharge and recharge may take place through the sedimentary cover. A group from the University of Washington, in a joint study with the Pacific Geoscience Centre in Canada, has made similar studies on the Juan de Fuca and Explorer ridges and found evidence for cellular convection and for the penetration of cold seawater beneath the sediments for distances of up to fifteen km from the nearest outcrops. Another survey on the flank of the Mid-Atlantic Ridge east of Brazil by the Lamont-Doherty group indicates that the crust is cooled up to twenty km from basement exposures requiring lateral flow rates in the range of 10^{-9} to 10^{-8} m/sec.

Particular attention has been paid to the two Low Heat-Flow areas of the Eastern

Pacific, discovered in 1963, by Von Herzen and Uyeda in the Guatemala Basin southwest of Central America and at the equator southeast of Hawaii. The areas are covered by thick pelagic sediments, 300m or more thick, yet their average heat-flow is less than one-third of the mean heat-flow from the earth. A Woods Hole group has now studied the Equatorial Low Heat-Flow Zone and found thermal evidence for an upward flow of porewater through the sedimentary blanket. The highest heat fluxes coincide with areas of rough basement which seem to focus the water flow.

Earlier this year, a Lamont-Doherty group made a detailed survey in the Guatemala Basin. Seismic profiling surveys showed a 400 m sedimentary blanket periodically broken by north-east trending ridges protruding through the cover and associated with fracture zones spaced 40 to 50 km apart. A line of temperature gradient measurements between two of the ridges shows anomalously low gradients even though the entire area between exposures is thickly sedimented. It appears that the basement in the Guatemala Basin is being 'ventilated' by this regularly spaced set of ridges.

During this survey, measurements of porewater pressure within the sediments were made by Dallas Abbott of the Lamont-Doherty Observatory. Anomalously, pore pressures are less than hydrostatic in sediments five meters below the seafloor and are accompanied by low

Marcus G. Langseth is in the Lamont-Doherty Geological Observatory, New York.

temperature gradients. Abbott explained that these are exactly the circumstances that would be expected if there were a downward flow of porewater. She suggested that the low temperature in the thick sediments of the Guatemala Basin may indicate a slow drawdown of seawater through the sedimentary cover that reemerges at the ridges.

Whether the flow through the sediments is, however, significant is controversial. Nonlinearities of temperature profiles on the ridge flanks have been repeatedly observed. The Lamont-Doherty group, on the basis of their 1977 survey, attributed these to water flow through the sediments. At most stations temperature gradients decrease with depth, implying upward flow.

The flow rates are surprisingly high, tens of centimeters per year. Even if the permeability of sediments is among the higher values found in the samples, pressure gradients of the same order of magnitude as the hydrostatic are needed to drive the flows. The pressures cannot be achieved by thermal stresses associated with thermal convection but three possibilities remain: 1) the *in situ* permeability is much higher than laboratory measurements indicate; 2) there are unknown sources of pressure in the oceanic crust; or 3) the non-linear temperature profiles may have another explanation. Nonlinearity in temperature profiles can stem from many phenomena; for example an increase in conductivity with depth, changes in the bottomwater temperature and heat generation in the sediments. The present data are insufficient to decide between alternative explanations and as a consequence, there is much interest in other approaches demonstrating significant flow of sedimentary interstitial water.

Chemical gradients in sedimentary porewater will also vary with depth if the flow rates are of the same magnitude as the ionic diffusion rates. A combined University of Rhode Island and Woods Hole study, reported at the June 1979 American Geophysical Union Conference, found calcium-depth profiles in sediments in the Galapagos Hydrothermal Mounds field consistent with an upward flux of 0.3 to 15 cm yr⁻¹; whereas, profiles in an adjacent low heat-flow zone show seawater concentrations of calcium consistent with downward advection of water. However, sediments in this area are only about 20-30 m thick.

Porewaters from DSDP cores provide chemical profiles across thick sedimentary layers and a consistent pattern is emerging from measurements made by geochemists at the Scripps Institute, Lamont-Doherty Observatory and the University of Rhode Island. Sediments in older areas of the ocean show chemical gradients that preclude water flow through the sediments at thermally significant rates. However, in ridge flank areas, chemical gradients in the sediments are small or nonexistent, so

vertical advection may be possible. The chemistry of interstitial waters promises to be one of the clearest means to detect water advection, particularly when coupled with temperature profiles.

The other means of investigating possible subsurface flows is by *in situ* pore pressure measurements. The measurements of Abbott in the Guatemala Basin were the first such experiments attempted in deep-sea sediments. The measurements are difficult to carry out but initial results are repeatable and show significant pressure anomalies. Further tests of this new technique are planned.

Deep-sea drilling on the Costa Rica Rift during DSDP-IPOD Leg 69 showed there was a substantial drop in excess pressure across a 260 m thick sedimentary layer. One hole drilled through the sediments and into the basement showed a drawdown of water of about one meter per hour which persisted for at least 60 days. Pore pressures at the bottom of this hole estimated from extrapolation of pulse tests were nearly twenty bars below hydrostatic. Anomalously low pressures below the sediments may be a widespread phenomenon, as they have already been reported at two sites in the Mid-Atlantic Ridge on Legs 37 and 46 and Leg 60 in the Mariana Trough.

There are a number of ways by which the low pressures may be produced. They include; a fossil low pressure, perhaps from the lowered sea level of the last ice age; alteration reactions in the basement that cause a volume decrease and large-scale thermal convection with the low pressure field centred below the sediments. The possibility that the low pressures result from intrabasement reactions suggests that inward flow of water at outcrops can occur without discharge, a result that would agree with seafloor heat-flow observations.

The proposal that Challenger be available for an additional two years of Deep Sea Drilling could allow new data to be obtained on the hydrology of the oceanic crust. Particularly important will be pressure measurements in holes that penetrate through sediments into igneous basement. These measurements can be made with a packer, a tool that seals the annulus between the hole wall and the pipe, allowing bottom-hole pressures and *in situ* permeabilities to be determined. Temperature and porewater chemistry observations in the sediments and in the bottom of holes that penetrate basement will add the third dimension to the seafloor distributions provided by conventional seafloor methods.

One of the most promising approaches to the problems discussed here would be to use the Challenger to reenter existing holes. These holes are in thermal equilibrium and the experiments using the packer could be efficiently done in a predrilled hole. A sufficient number of reentry holes in both the ridge flank and in older seafloor exist to permit a variety of thermal and sedimentary environments to be sampled.

The questions about the extent and magnitude of circulation in the suboceanic crust have great relevance to problems of nuclear waste disposal in the deep seabed. The US Department of Energy is considering the burial of waste in durable canisters within sediments such as the abyssal clays of the Northern Pacific which are assumed to be impermeable. Incomplete knowledge of the driving forces and extent of water circulation in the crust and sediments makes these assumptions questionable. However, should it turn out that low pressures in the oceanic crust are not associated with circulation that returns seawater to the ocean, then long-term disposal may become a possibility. □

Membrane protein distributors

from M.J. Geisow

ON theoretical as well as experimental grounds, lipid vesicles have long been considered the likely carriers of proteins between the membranes of the cell. Rothman and Fine (*Proc. natn. Acad. Sci. U.S.A.* 77, 780; 1980) have now demonstrated that a trans-bilayer glycoprotein, the small G protein of Vesicular Stomatitis Virus (VSV) is inserted into the membrane of coated vesicles during its migration to the surface of CHO cells. Furthermore — and of wide importance for studies of the transport of proteins synthesised on rough endoplasmic reticulum (RER) — intracellular transit of G from RER to the Golgi apparatus in which terminal sugars are added also takes place in clathrin-coated vesicles.

M.J. Geisow is in the National Institute for Medical Research, Mill Hill, London.

The character of the clathrin coat and its self-assembly in the plasma membrane may provide a basis for the specificity required in a membrane protein transport system. As early as the 1960's, Friend and Farquhar (*J. Cell. Biol.* 35, 369; 1967) suggested that coated vesicles conveyed material to apical regions of the plasma membrane in rat vas deferens epithelial cells. The vesicles were seen in close association with the Golgi apparatus and cytochemical observations suggested that they might also transport lytic enzymes to lysosomes but supporting biochemical evidence was lacking until the experiments of Rothman and Fine.

Their choice of a viral protein to track the route of membrane proteins was a simplifying one as G is the only glycoprotein synthesised in CHO cells following VSV infection. In just the same way as native membrane glycoproteins, G

is glycosylated in the endoplasmic reticulum and hexamannose units of the core oligosaccharide are replaced by terminal sugars in the Golgi apparatus. The pre-Golgi form of G is distinguishable from the post-Golgi processed protein by its sensitivity to endoglycosidase H (Endo H).

Coated vesicles purified from a large pool of VSV-infected cells contained G as well as the characteristic spectrum of vesicle polypeptides and G could be co-precipitated with clathrin using mono-specific anticlathrin. Newly-synthesized G was detectable by pulse-chase labelling, in this case carrier vesicles derived from bovine brain allowed isolation of a small number containing radiolabelled G. A kinetic study of the transport of G in the vesicles was then possible, using Endo H to determine the amount of Golgi-processed protein.

It was clear that G was present in coated vesicles in transit to the plasma membrane since the vesicle fraction never contained more than 20% of the pool of labelled protein. More importantly, the specific activity of labelled G in the vesicles rose twice during the chase, showing the transport of first the Endo-H sensitive (pre-Golgi) glycoprotein and then the transport of the Endo-H resistant glycoprotein. The clearance of the post-Golgi form of G from the vesicles coincided with the appearance of G on the cell surface (55% of the label in 65 min).

G was demonstrated to be actually present within the coated vesicle membrane by uncoating vesicles containing ^3H -mannose-labelled G on metrizamide gradients. A single ^3H -labelled polypeptide corresponding to G floated up with the CV lipid vesicle fraction.

Coated vesicles are known to 'shuttle' rapidly within cells and short transit times are certainly in keeping with their functions as a transport system. The presumed cycling time of coated vesicles is much faster than the observed time for the total transport of pulse-labelled VSV G protein suggesting that many trips are made by the same vesicles or at least certain of their components. The difficulty of depleting some endocytic receptors which are known to be internalized in coated vesicles again suggests that they are re-used.

Of course, the recognition of the identity of the carriers still leaves open the question of how a given protein arrives at the appropriate cellular membrane, but at least this sorting problem has been brought nearer to the compass of current experiment.

Adhesion of pathogenic microorganisms

from Mary Lindley

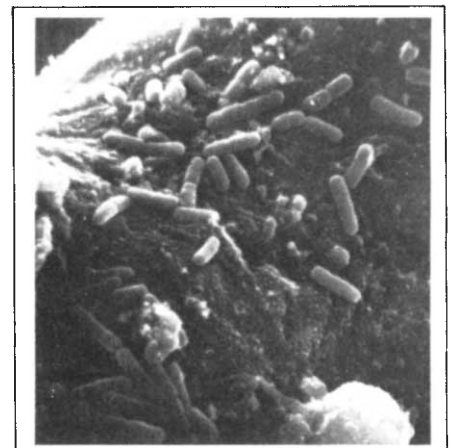
MICROORGANISMS that enter the internal tracts of the body and come to rest on mucous membranes would seem to need a firm anchorage before invading or discharging their toxins into the underlying epithelium. Otherwise they would surely be swept away by the muscular activity of the tract or by the fluid passing through. Nevertheless, a link between adhesion and pathogenicity was recognised only slowly. The first clear evidence of the significance of bacterial adhesion came during the 1970s when veterinary scientists showed that certain strains of *Escherichia coli* must produce not only toxins but also an antigen, designated K88, in order to cause diarrhoea in piglets. Recognised by the ability to agglutinate red blood cells, K88 and the similar K99, produced by strains infecting calves and lambs, are hair-like fimbriae (also known as pili but unrelated to sex pili), which mediate adhesion to the intestinal mucosa. Analogous plasmid-encoded fimbriae, designated colonisation factor I and II (CFA/I and /II), have been described in certain *E. coli* from cases of human diarrhoea.

Thus a similar picture has begun to emerge for human diarrhoea, although the pace of work is hampered by the difficulty of finding a suitable system to study. At a recent symposium on adhesion and pathogenicity* D.C.A. Candy (Institute of Child Health, London) reported tests of adhesion of *E. coli* to human buccal epithelial cells and intestinal cells. This work has confirmed that CFA/I is important in adhesion to human small intestine. Investigation of an outbreak of acute diarrhoea in infants has led to the isolation of a strain of *E. coli* producing a colonisation factor resembling CFA/II.

There are also signs that some infants with protracted diarrhoea may be more prone than others to adhesion by *E. coli*, in the same way that some but not all piglets develop diarrhoea in response to *E. coli* carrying K88.

Apart from CFA/I and /II, strains of *E. coli* causing diarrhoea in man may carry antigens of a different sort — type 1 fimbriae (also known as common fimbriae). M.M. Levine and his colleagues (University of Maryland School of Medicine, Baltimore) are looking for a combination of all three that could form the basis of vaccine against infant and travellers' diarrhoea, analogous to that already used successfully for piglets. He suggested that a vaccine would probably include antigens such as CFA/I and /II plus type 1 fimbriae of perhaps three different antigenic varieties. The immediate task is to find antigens common to a sufficient variety of bacterial strains to provide a broad spectrum of protective antibodies when used as a vaccine.

Studies of *Neisseria gonorrhoeae* adhering to buccal epithelial cells, described by E.C. Tramont (Walter Reed Army Medical Center, Washington DC) have shown that fimbriae are the principal antigens mediating adhesion, again making them the obvious target for prophylaxis. Human antibodies against these fimbriae can be found in genital secretions from naturally occurring cases of gonorrhoea, albeit needed in very large amounts to block adhesion *in vitro*. One possible approach to prevention could be immunization to produce protective antibodies against fimbriae. The other approach discussed by Tramont would be to inhibit adhesion competitively by bathing the site of infection in fimbriae. Tramont believes that any vaccine against

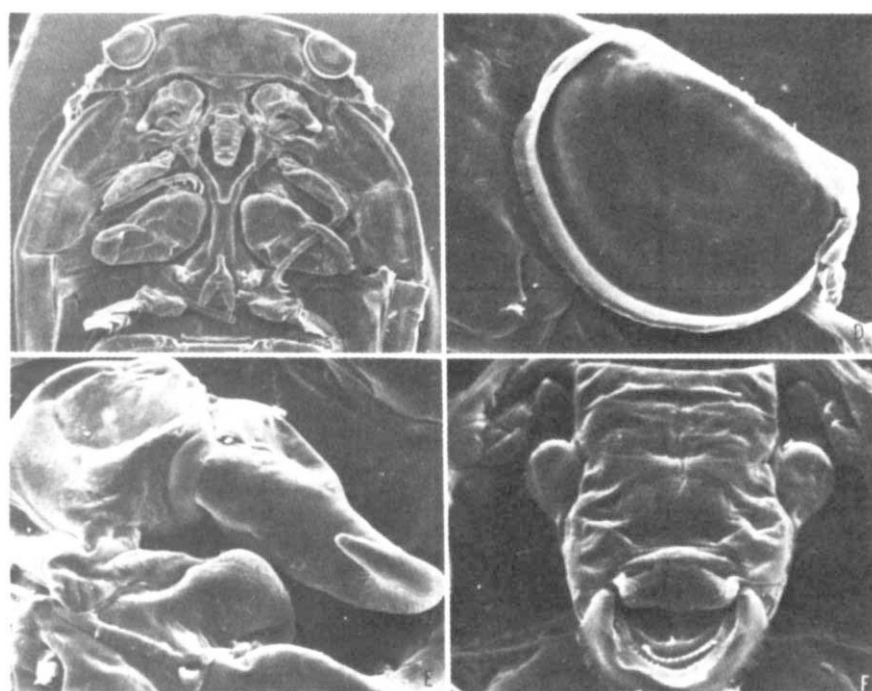


Enteropathogenic bacteria (*E. coli*:026:K60) adhering to human small intestine. (Photograph: A. Phillips, Queen Elizabeth Hospital, London E2.)

gonorrhoea will have to be applied locally to give the best possible results.

C. Svanborg Edén (University of Göteborg, Sweden) thinks that fimbriae also mediate the adhesion of those intestinal *E. coli* that infect the urinary tract. She has found that the extent of adhesion seems to be related to virulence, which is greatest in strains causing acute pyelonephritis, intermediate in those causing acute cystitis, and low in those producing no symptoms although they are detectable in the urine. Susceptibility to infection also seems to vary, and this, she suggested, may be associated with the density of receptors on the host cells. Candidates for those receptors are glycolipids related to globoside, which inhibit the adhesion of *E. coli* to urinary

*A symposium on Adhesion and Microorganism Pathogenicity was held on 13-15 May at the Ciba Foundation in London. It was organised by Dr Katherine Elliott and the proceedings will be published by Pitman Medical.



Caligus coryphaenae Steenstrup and Lütken, female: top left, cephalon, ventral (30X); top right, frontal lunule (200X); bottom left, second antenna (200X); bottom right, a happy mouth tube (280X).

from R.J. Roberts

A recent Smithsonian Contribution to Zoology* by Cressey & Cressey has made a major contribution to resolving many of the problems of copepod taxonomy. There are more than a 1,000 known copepod species with very wide ranging structural diversity and the same hallowed, often inaccurate, drawings have appeared in text after text. The copepods are parasites of the economically important mackerel and tuna fish groups and when they occur in large numbers they can be a source of significant financial loss.

The scanning electron microscope pictures from the report, reproduced alongside, show various features of a parasite of the tunny. The bottom right-hand plate, which they designate "the happy mouthtube" shows something that parasitologists have long suspected — that copepods enjoy life even if their hosts do not.

R.J. Roberts is Director of the Institute of Aquaculture, University of Stirling, Scotland.

*Roger Cressey & Hilary Boyle Cressey *Parasitic Copepods of Mackerel- and Tuna-like Fishes (Scombridae)* of the World Smithsonian Contributions to Zoology 311 (Smithsonian Institution Press, City of Washington, 1980).

tract epithelium, whereas other glycolipids and monosaccharides do not.

N. Sharon (Weizmann Institute, Rehovoth) reminded the symposium that bacterial adhesion may be inhibited by simple sugars, particularly mannose in the case of various strains of *E. coli* and salmonella. This has suggested that mannose or mannose-like residues on the host cell form part of the receptor for the bacteria which attach to them. There is considerable evidence, said Sharon, that many strains of bacteria contain a lectin-like substance, specific for mannose, which may take the form of fimbriae or flagella or may be embedded in the outer membrane. (Adhesion mediated by CFA/I and II, K88 and K99 is insensitive to mannose, while that due to type 1 fimbriae is sensitive.) He said there is good reason to believe that the mannose-sensitive attachment plays a role in pathogenicity, and he has found that α methyl-D-mannoside diminishes infection of the urinary tract of mice by virulent strains of *E. coli*.

Adhesion, often of a more transitory nature, is an obligatory step in the infection of cells by mycoplasma. S. Razin (Hebrew University of Jerusalem), describing work done in conjunction with W. Bredt (University of Freiburg), said that adhesion, chiefly in the respiratory tract, enables toxic products of mycoplasma metabolism, such as ammonia and hydrogen peroxide, to reach concentrations sufficient to damage the cells. He said that sialic acid residues form the receptors of the cells, while the binding sites on the mycoplasma are apparently membrane proteins. Adhesion seems to

depend on the metabolic state of the mycoplasma, with the electrochemical ion gradient across the membrane also being influential.

Another parasite, the protozoan responsible for amoebic dysentery, has come under closer scrutiny as a health problem of the developing world. The trophozoite stage of *Entamoeba histolytica* begins the infectious process by adhering to intestinal epithelial cells, and from there can penetrate into the circulation, adhering to red blood cells and phagocytosing them. D. Mirelman (Weizmann Institute) reported that a lectin found in the trophozoite is apparently involved in the adhesion. He thinks the lectin is separate from the amoeba's toxin, for the two activities can be demonstrated in different fractions obtained from the trophozoite.

For the merozoite stage of the malaria parasite, adhesion to red blood cells is an essential part of the cycle of invasion, multiplication, release and reinvasion that produces characteristic symptoms of anaemia and fever in the host. Adhesion seems to be mediated by specific receptor molecules on merozoite and red cell — evidence includes the specificity of each species of merozoite for either young or old red cells of certain host species and for a particular blood group antigen. R.J. Howard (National Institute of Allergy and Infectious Diseases, Bethesda), discussing work with *Plasmodium vivax* and *Plasmodium knowlesi*, pointed out that characterization of the molecules involved in recognition is hampered because the morphological changes occurring during the cycle are likely to affect the receptors.

D.F.H. Wallach (Tufts-New England Medical Center, Boston) recalled that after invasion, a merozoite matures and multiplies within a vacuole. At the same time the host red cell's cytoskeleton is destroyed and its surface membrane is altered by modification of component proteins and insertion of others synthesised by the parasite. The new proteins are immunogenic in the host, and two of them are involved in protective immunity and have antigenic features common to several species of *Plasmodium* that cause malaria in mammals. This lack of species specificity should make such proteins an attractive target in the search for a vaccine against malaria.

Although the details of adhesion and its relationship to pathogenicity are likely to be hard won, the potential rewards in terms of prevention and treatment of disease are clearly considerable. Vaccines are an obvious outcome to hope for, but other approaches are possible. E.H. Beachey (University of Tennessee College of Medicine, Memphis) reported that small, sublethal concentrations of various antibiotics can disrupt the adhesion of some *E. coli* and *Streptococcus pyogenes*. Especially effective are antibiotics that act on the ribosomes or the cell wall, and Beachey presumes that they interfere with the synthesis or assembly of the fimbriae. This suggests the possibility of combating bacteria with doses of antibiotics sufficient to prevent adhesion but not necessarily to kill them; such low doses would avoid problems of toxicity. As the details of adhesion become better understood, more such relationships should emerge.

Virus crystallography comes of age

from Stephen C. Harrison

VIRUS particles are simple paradigms for all sorts of sub-cellular assemblies, and detailed views of their organization have long been sought. X-ray diffraction studies of virus crystals began at almost the same time as crystallographic investigation of small proteins¹, and a certain mystique of the 'ultimate' in large unit cells grew up around them. The reports in *Nature* by Abad-Zapatero *et al.*² of Southern Bean Mosaic Virus (SBMV) at 2.8 Å resolution and by Unge *et al.*³ of Satellite Tobacco Necrosis Virus (STNV) at 4 Å resolution follow by less than two years the publication of 2.8 Å resolution structures of Tobacco Mosaic Virus (TMV) disk⁴ and of Tomato Bushy Stunt Virus (TBSV)⁵: 'virus crystallography' is no longer a vague promise, and several striking results emerge from comparison of the structures at hand.

All these efforts have relied on some recent advances in technique — particularly in computing. Rossmann and Blow⁶ observed almost 20 years ago that non-crystallographic symmetry in structures such as viruses could enormously facilitate solution of the X-ray phase problem, but only in the last five years have algorithms, machines and

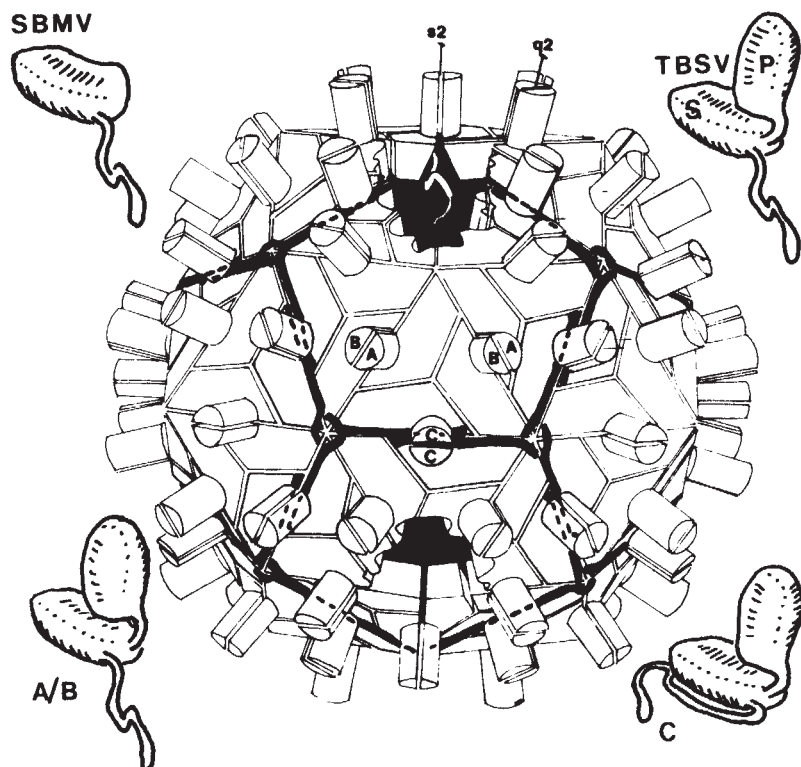
programs been available to carry out the lengthy iterative calculations⁷. In principle, these iterations should converge from any initial approximation to the phases; in practice, however, experimental errors in observed structure amplitudes and approximations in the computational scheme require a non-random starting point. The work on TMV disk, TBSV and SBMV have all relied on the standard method of isomorphous replacement to provide this start. The STNV solution is rather more daring, using isomorphous phases to 10 Å and phase extension stepwise to 4 Å by non-crystallographic symmetry averaging. A more conventional computation was also performed, using initial SIR phases to 4 Å, and the reported agreement between this and the phase-extension calculation is excellent. It is difficult to assess the accuracy of a 4 Å map from density alone, since the shape of features depends on local conformation, and a more complete measure of the success of phase extension will be recognition of polypeptide chain and residues when the STNV structure is carried to 3 Å or beyond.

The coat subunit of a spherical virus assembles into a regular, icosahedrally symmetric shell and the shell is a package

for nucleic acid⁸. Moreover, in most structures the same subunit is found in several conformationally distinct positions, genetic economy requiring a larger coat than can be constructed from just sixty (i.e. one icosahedral quota). These architectural conditions demand a way of incorporating nucleic acid that adapts an inherently nonrepeating structure (the loops and stems of folded RNA) to a regular surface lattice. In structures with more than 60 subunits, they further demand a subunit that can adopt alternative bonding arrangements and hence also a mechanism for avoiding ambiguity.

In TMV, which has been resolved at 4 Å by diffraction from orientated gels⁹, three nucleotides are bound into a groove-like site on each subunit. The coat is an 'unwinding protein' and imposes its own helical order on the RNA backbone. One might imagine by analogy that each protein subunit of a spherical virus could bind to an exact length of nucleic acid. If the binding sites were located on a part of the protein fixed in the surface lattice, the RNA would be piecewise icosahedrally ordered, and portions of it would be visible in a suitable electron density map. This model can be ruled out in TBSV and apparently now in SBMV, where no clear RNA-related features appear. In both cases, however, a significant portion of the protein subunit (about 60 residues) is also invisible in the map, implying absence of a unique spatial relationship to the rest of the chain. This portion lies at the N-terminus, and it is a plausible candidate for a nucleic-acid binding sequence. If so, we can imagine two RNA packing modes that might interact with a flexibly-linked binding domain: (1) Viral RNA *in situ* could have a more or less defined tertiary structure, requiring binding regions on the protein loosely tethered to the rest of the subunit, in order to seek out appropriate sites on a non-icosahedrally-symmetric nucleic-acid structure; (2) RNA stems and loops could be compactly but essentially randomly close-packed in the particle (some 60 to 70% of the RNA in such viruses is probably in double-helical stems¹⁰), again requiring flexibly-linked protein regions for interaction with appropriate RNA structures or sequences. Crystallographic structure determination necessarily yields an average over the operations of the space group, and in SBMV and TBSV, a unique RNA structure would be blurred by the high symmetry of the lattice position on which the particle lies. In STNV, the asymmetric unit comprises an entire particle, and Unge *et al.* point out that

Subunits and their arrangement in SBMV and TBSV. The SBMV subunit (upper left) has an N-terminal arm and a single, folded domain, homologous to the S-domain of TBSV (upper right); 180 subunits pack to form a T = 3 viral shell. The arms of 60 of the subunits (positions denoted C) are ordered as shown by heavy lines in the central diagram. The actual folding of an ordered arm is along an inside edge of the S-domain with a loop around the particle threefold axis (see diagram in lower right-hand corner). Three such loops embrace, and the 60 ordered arms form a connected framework. The arms of subunits in positions denoted A and B are not regularly ordered in SBMV or TBSV (lower left). The cylindrical projections in the central diagram represent P-domain dimers in TBSV; they are, of course, absent in SBMV.



Stephen C. Harrison is Professor of Biochemistry at Harvard University.

crystallographic symmetry alone does not exclude determination of RNA configuration. Such a determination would require not only a unique RNA conformation, but also sufficient asymmetry imposed thereby on the shell that consistent orientations appeared throughout the crystal. The present map is ambiguous on that point, but a high degree of order does not appear likely.

The N-terminal 15 residues cannot be traced, and the general appearance of internal density is not immediately recognizable as a single structure. It is useful in this connection to recall that the uniqueness of the secondary structure has not been established for viral RNA, although the correlation of nuclease sites and predicted single-stranded regions in 'flower diagrams' of the MS2 sequence suggest a general regularity¹¹. In any one stock of virus, a given RNA molecule is likely to differ from the mean sequence at a number of positions¹². Moreover, the compactness of the configuration in the virus appears to be imposed by the shell, rather than by its own sequence, since RNA of the size of the genomes in these viruses has a much larger radius of gyration under conditions of physiological ionic strength than that of the cavity into which it is packed¹³. Indeed, the degree of compaction is striking: from figures given by Unge *et al.*, the volume available to STNV RNA implies an inner cavity with space for about 1.2 g H₂O per g RNA; the packing of TBSV RNA is comparably tight. A model of more or less random close packing of roughly parallel RNA stems is appealing, since very efficient packaging can be achieved without any significant demands on RNA structure or sequence. But it will not be simple to prove such a picture.

The most surprising of recent results is probably the similarity of the SBMV subunit to the S-domain of TBSV. Various evolutionary speculations can be made, but

from the point of view of assembly and its control, there are two more immediate conclusions that should be emphasized. The first is the identical way in which these two T=3 structures solve the ambiguity problem alluded to above. A portion of the polypeptide chain, just N-terminal to the compact domain(s), is ordered on sixty subunits and disordered on the remaining 120. The sixty ordered arms effectively serve as an internal, T=1 framework: they interdigitate around icosahedral twofolds. This scaffold, formed by a flexibly linked part of the subunit chain determines the size and curvature of the particle. The choice between alternative bonding patterns at interfaces that must so adapt is dictated by the symmetry and geometry of the network of arms. One might imagine cases where the network would be formed of a distinct protein — could this be the case with cores in bacteriophage head assembly¹⁴ or VP2 and VP3 in polyoma and SV40¹⁵? The second direct conclusion that emerges from comparison of TBSV and SBMV is the significance of modularity in the design of assembling subunits. In SBMV as seen in the paper of Abad-Zapatero *et al.*, the subunit has three functional parts: an unseen (RNA-binding?) N-terminal piece, the framework-like arm (including sequences in the intertwined β -annulus), and the major folded domain. In TBSV, there is an additional folded domain (the P-domain).

Clearly the P-domain is not essential for particle stability, since it is missing in SBMV. It does appear to be a strong twofold 'clamp', and the hinge between it and the S-domain is required for subunits to conserve these two fold interactions while still adapting to the T=3 surface lattice.

With these advances now at hand, what about the crystallography of other viruses and large assemblies? SBMV and TBSV show quite dramatically that the spatial arrangement of modules in a subunit protein is characteristic of the assembly, but not necessarily of the isolated polypeptide; the remarkable interdigitation of N-terminal arms could not have been anticipated (or even seen) in the structure of the subunit alone. The possible consequences for those who would like to visualize a ribosome, for example, are sobering: high-resolution structures of large assemblies may not readily be constructed from structures of component parts. There is, however, a converse advantage of flexibly-linked modules: in cases where function is a property of a particular module of a large protein, proteolytic dissection and piecemeal crystallization is a fruitful and helpful strategy. This is the case, for example, with influenza virus haemagglutinin¹⁶ and may well be appropriate for the presumptive RNA-binding, N-terminal portions of SBMV or TBSV. □

Open-ended fusion systems

from R. F. Post

JAPAN, among all of the highly industrialized nations, is the most dependent on imported energy. Japan is also a nation that owes its advanced technology both its present economic strength and its hopes for future economic growth. Given these circumstances it is not surprising that nuclear fusion research has been singled out for special attention by the Japanese government. Fusion, for which the primary fuel is heavy hydrogen, extractable at low cost from water, would be an extremely attractive long-term energy source. It is against this backdrop that a specialized scientific meeting dealing with a particular approach to fusion, so-called open-ended magnetic confinement systems was convened in Japan in April.*

Open-ended fusion systems are a class of fusion 'magnetic bottles' in which the field lines of the externally-generated magnetic field (needed to confine the hot fusion plasma) enter at one end of the bottle and exit from the other end. Open-ended magnetic bottles are in contrast to ones such as the tokamak, where the field lines remain entirely within the doughnut-shaped confinement chamber.

R. F. Post is Deputy Associate Director for Physics in the Magnetic Fusion Division of the Lawrence Livermore National Laboratory, University of California.

An example of an open-ended system is the simple mirror machine, in which the field lines form a tubular bundle constricted at both ends (the mirrors). The charged particles of a fusion plasma, once trapped between the mirrors, continue to gyrate back and forth along the field lines, being repeatedly reflected by magnetic mirrors in the same way that charged particles are trapped, between the north and south magnetic poles, in the Van Allen radiation belts that surround the earth.

Present interest in the mirror approach to fusion power stems in part from an inherent advantage that some kinds of mirror fields possess in their ability to suppress the so-called MHD or fluid-like turbulences in magnetically confined plasmas. From this property derives the remarkable ability of specially-shaped mirror fields (called 'magnetic wells') to stably confine very high plasma pressures for a given magnetic field intensity. To this inherent advantage has recently been added a new feature — the tandem mirror idea — an idea that shows promise of greatly improving the economic prospects of mirror fusion systems relative to older mirror approaches. The tandem mirror

The 'International Symposium on Physics in Open-Ended Fusion Systems' was held at the new University of Tsukuba, Japan in April 1980.

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consists of two small conventional mirror cells located at each end of a large central confinement chamber around which is wound a simple solenoidal magnet coil. The hot plasmas in these end cells create positive electric potentials that act to electrostatically 'plug' the end leakage of charged particles from the central cell far more effectively than is possible with a single mirror at each end acting alone. Though invented in the USSR and, independently, in the US about four years ago, the first tandem mirror device to be constructed and operated (Gamma-6; 1978) was built at Tsukuba University, Japan, preceding the larger experiment (TMX; 1979) brought on line at the Lawrence Livermore Laboratory.

The scientific validity of the tandem mirror idea for enhancing mirror confinement was made clear in papers by Kawabe (Tsukuba) and Grubb and Drake (Lawrence Livermore Laboratory) who described experiments in the Tsukuba Gamma-6 and Livermore TMX experiments, respectively. Work in progress (TMX) includes more detailed measurements of particle and energy transport, the role of microinstabilities and their suppression, and the study of plasma pressure limits as determined by MHD effects. Baldwin (Livermore) then described recent developments in tandem mirror theory, including the important new idea (originated by Baldwin and Logan) of the thermal barrier. In a tandem mirror, a thermal barrier is a specially designed cell that, through the generation of a locally-negative potential region, can act to thermally isolate the end plug from the cen-

tral cell. Thermal isolation then would permit the heating of plug electrons to higher temperatures than those of the central cell, with resultant increased plug potential at lowered plug plasma density — a major economic advantage.

The use of radiofrequency fields to enhance the confinement of open-ended systems has been a major topic in fusion research in Japan for many years. Pioneered at Nagoya under the direction of K. Takayama, this work led to the construction at Nagoya of a large mirror-cusp device, RFC-XX. Itatani (Nagoya) described preliminary results with this machine, in which enhancement of confinement of moderate temperature plasmas by factors of 5 or more (over the confinement observed with r.f. turned off) were seen. Whether good confinement of higher temperature plasmas can be achieved is not yet resolved. RFC-XX however appears to be the most advanced facility for the study of such questions in any fusion laboratory.

An important sub-class of mirror systems are those where the phenomenon of field-reversal is invoked to improve confinement. In a field-reversed mirror — a form of the so-called "compact toroid" configuration — the internal diamagnetic currents of the mirror-trapped plasma are made to be sufficiently intense to reverse the direction of the magnetic field near the axis within the plasma, thus closing the magnetic lines on themselves within the plasma interior and thereby inhibiting plasma losses through the mirrors. Interest in such configurations, which dates back many years to related systems (such as the

electron ring ASTRON of Christofilos), comes from their geometric simplicity and, especially, from their extremely efficient use of the external field. The field-reversed mirror shares with the tandem mirror the generic idea that properties of the plasma are used to improve its own confinement. The Symposium provided a forum for the discussion of most of the FRM work now in progress, including, for example, work at the Los Alamos Laboratory on field-reversed configurations produced by a coaxial gun (Sherwood; Los Alamos) and field-reversed electron rings (Fleischmann; Cornell).

Some of the most unusual mirror devices were described in the papers concerning work at Novosibirsk (Rytov, Koidan). In addition to the AMBAL tandem mirror, now under construction, there are two other mirror devices: PSP-2, a device in which mirror confinement is enhanced by electrically-induced plasma rotation, and GOL-1, a device using multiple mirror cells and operating at very high plasma density. Both of the latter innovative devices owe much in their origin to the former Director of the Novosibirsk fusion group, G. I. Budker, who died July 4, 1977.

Judging from the lively discussion at the conference, and from the enthusiasm of the participants, interest in open-ended systems is increasing not only in Japan but world wide. In the case of the Japanese effort, it seems equally clear that their contributions to research on open-ended magnetic fusion systems will become increasingly important over the next few years.



100 years ago

PHYSICS WITHOUT APPARATUS

It is almost a proverb in science that some of the greatest discoveries have been made by the most simple means. It is equally true that almost all the more important facts and laws of the physical sciences can be illustrated and explained by the help of experiments made without special or expensive apparatus, and requiring only the familiar objects of common life for their performance. The greatest exponents of popular science — and amongst them notably Faraday — delighted in impromptu devices of this kind. It is indeed surprising how throughout the whole range of natural philosophy the hand of the master can turn to account the very simplest and rudest of apparatus. A silver spoon, a pair of spectacle lenses, a tumbler of water, and a few sheets of paper suffice to illustrate half the laws of geometrical optics. A few pieces of sealing-wax, some flannel, silk,

writing paper, pins, and glass tumblers will carry the clever experimenter a long way into the phenomena of electricity. These are things which any person can procure, and which any person can be taught to use. But their right use depends on the possession of



accurate scientific knowledge and a clear understanding of *what* the various experiments are to prove. In fact the art of experiment and the science of inductive reasoning are the essential qualifications necessary to make *Physics without apparatus* profitable.

Amongst the simple mechanical laws with which a beginner in physics must acquaint himself is that commonly referred to as the *law of inertia*, which is, however, very often so imperfectly expressed as to be misapprehended. It requires force to move matter, not because matter is inherently lazy or sluggish,

but because it possesses *mass*. The greater the mass of matter in a ball, the harder work is it to send it rolling. Force is also required to stop matter that is moving, the reason again being that a mass moving under the impulse of an impressed force possesses a certain moving energy which cannot be at once reduced to nothing. In either case — either to move a mass or to alter the motion of a mass — force must be employed and energy expended. Of this law of inertia many examples might be given: and there are many curious facts which this law serves to explain. Some of the most striking of these are those in which the effect of sudden forces is different from that which might have been expected. In Fig. 1 we give an illustration of an experiment of this nature. A wooden rod — say a broomstick — has a couple of needles fixed into its ends, and it is then supported upon two wineglasses resting upon two chairs. If a heavy poker is now brought down very violently upon the middle of the stick it will break in two without the needles or the glasses being broken. A feeble or indecisive blow will fail to do this, and will break the glasses or the needles, or both. Here the moving energy of the heavy mass, the poker, is suddenly transferred to the middle of the stick, so suddenly that it is broken asunder before the thrust has time to reach the fragile supports. From *Nature* 22 5 August, 320 & 321, 1880.

REVIEW ARTICLE

New findings in methane-utilizing bacteria highlight their importance in the biosphere and their commercial potential

I. J. Higgins, D. J. Best & R. C. Hammond

Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, UK

Recent results, showing that the ubiquitous methane-utilizing bacteria (methanotrophs) can partially oxidize and, in some cases, extensively metabolize complex organic compounds, call for a reappraisal of their role in the cycling of elements in the biosphere. Possible environmental implications and opportunities for industrial exploitation are discussed.

ABOUT half of the substantial, but not precisely known, proportion of total organic carbon degraded by anaerobic microflora is converted to methane. The amount of the hydrocarbon released into the atmosphere, however, represents only about 0.5% of total carbon turnover¹. The disparity between this figure and the large amounts of methane produced by the methanogenic bacteria is due mainly to the activities of aerobic methane-utilizing microorganisms (methanotrophs), situated between the anaerobic sediments responsible for methane production and the atmosphere, and partly to the microbial oxidation of methane anaerobically. On this basis alone, organisms responsible for the oxidation of methane are therefore of major importance in the carbon cycle and much current research effort is being devoted to an understanding of their physiology and biochemistry. Our present knowledge has been comprehensively reviewed in recent years²⁻⁶ and our purpose here is not to reiterate this information but rather to survey important new findings implicating methane-oxidizing bacteria in transformations of substances which are not their growth substrates, particularly multi-carbon compounds, and to highlight possible consequences and applications of these microbial activities.

The ubiquity of methane-utilizing bacteria is attested to by the variety of sources from which various workers have been able to isolate them⁷. The many strains isolated in the last 15 years are predominantly aerobic Gram-negative bacteria, growing only on methane or, in some cases, on methanol or dimethyl ether. They can be divided into two distinct groups (types I and II) based on the morphology of their internal membranes and the nature of their carbon assimilation pathways. In recent years, however, facultative methane-oxidizing bacteria, apparently similar to type II obligate methanotrophs, have been isolated^{8,9} and some yeasts have been implicated in methane oxidation¹⁰. Evidence for the involvement of fungi and algae in this process is more equivocal. Although microbial utilization of methane is primarily an aerobic process, it has been shown recently that some species can oxidize it anaerobically. In particular, energy for growth can be obtained by coupling methane oxidation to carbon dioxide with sulphate reduction to hydrogen sulphide¹¹. A small amount of methane oxidation may also be accomplished by methanogens¹².

Mechanisms of methane oxidation

In the current context we are particularly concerned with the aerobic, obligate methanotrophs because of recent advances in our understanding of the mechanisms of methane oxidation in these bacteria. It has been known for many years that these microorganisms oxidize methane to carbon dioxide by a route involving successive two-electron oxidation steps via methanol,

formaldehyde and formate. Carbon is incorporated into cell material at the oxidation level of formaldehyde by type I species, which use the ribulose monophosphate pathway (Quayle cycle), and in type II species, using the serine pathway, as formaldehyde and carbon dioxide²⁻⁶. Both groups of bacteria can grow only on a restricted range of reduced C₁ compounds and, in their selectiveness resemble the so-called obligate autotrophs. Nevertheless, it has long been known that such bacteria, either as washed suspensions or in culture, will partially oxidize simple substrate analogues, such as ethane, propane and butane, to the corresponding alcohols, aldehydes and fatty acids¹³. This was presumed to reflect some lack of specificity in the enzymes involved in methane oxidation. Subsequently, it was shown that carbon monoxide, ammonia and ethene are also oxidized¹⁴⁻¹⁶. However, there is now known to be a surprisingly vast range of multi-carbon compounds, often not closely related to the natural substrates, which are oxidized by methanotrophs¹⁷⁻¹⁹. Although this capacity varies between species, the following types of compound are oxidized by washed cell suspensions: long-chain alkanes (up to at least hexadecane), alkenes, aromatic and alicyclic hydrocarbons, phenols, long-chain and alicyclic alcohols, pyridine, multi-ring compounds and chlorinated aromatic hydrocarbons. In each case only a limited number of products (sometimes only one) are formed as a result of this unexpected activity, showing that there is, nevertheless, some mechanistic specificity. In some cases the products are simply hydroxylated derivatives, suggesting that a reaction analogous to the oxidation of methane to methanol has occurred. Commonly, there is further oxidation of these hydroxylated compounds to yield aldehydes and carboxylic acids. Many of these biotransformations have been shown to occur in both the presence and absence of methane¹⁷.

'Fortuitous metabolism'?

These activities have been described as 'fortuitous metabolism' (ref. 18), implying that they are merely due to the lack of specificity of oxidative enzymes, whose primary function is to effect the oxidation of methane to carbon dioxide. Such extraordinary lack of enzyme specificity would be extremely unusual if it were entirely fortuitous and we argue here that this phenomenon has developed and been retained because of its survival value to these species. It may well also be of environmental importance and could prove of commercial value, as evidenced by the recent filing of a number of patents on this subject²⁰⁻²⁶.

We now have some understanding of the enzymological basis of this lack of specificity and it mainly reflects the properties of enzymes initiating methane oxidation, methane monooxygenases (MMOs). In common with some other

monooxygenases, these enzymes have proved difficult to purify but oxygenase involvement was clearly indicated by the ^{18}O -incorporation experiments described by Higgins and Quayle²⁷. Subsequently, cell-free activities were studied in a number of species, but substantial purification has been reported in only two, *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b. The system seems to be best characterized in the former species and there is some uncertainty concerning the true nature of the system in the latter^{3,4}. There is evidence, however, that growth, and perhaps storage conditions, may markedly affect the characteristics of the enzyme of *M. trichosporium*²⁸. Since the first report of cell-free MMO activity by Ribbons and Michalover²⁹, several workers have reported that such activities are not totally specific for methane, higher gaseous alkanes, alkenes, ammonia and carbon monoxide also being oxidized³⁰⁻³³. The range of substrates for MMOs was dramatically extended by Colby *et al.*³⁴, who showed that cell-free extracts of *M. capsulatus* (Bath) oxidized a wide range of hydrocarbons and related compounds. It has since been shown that enzymes from other species are also relatively nonspecific, but in some cases the specificity is more restricted¹⁸. Although these findings account for the ability of whole organisms of some methanotrophs to introduce an oxygen atom into many compounds, they do not explain the further oxidation of some of these initial products to, for example, aldehydes and carboxylic acids. These further oxidation processes for simple analogues of methane no doubt reflect lack of absolute specificity of the methanol- and formaldehyde-oxidizing enzyme systems. In the case of more complex hydroxylated products, recent evidence suggests the involvement of other enzymes with no known roles in C_1 metabolism. For example, a number of species contain a broad-specificity, secondary alcohol dehydrogenase³⁵. It was thought that intact cells of *M. capsulatus* (Bath) had a rather limited oxidizing capacity¹⁸ but it is now known to be more extensive (H. Dalton, personal communication).

Transformations effected by *M. trichosporium*

Recent work in our laboratory has revealed not only that intact *M. trichosporium* can effect the partial oxidation of a relatively wide range of hydrophobic organic compounds but also that the transformations, in some cases, involve more extensive or even non-oxidative metabolism¹⁷. Examples of transformations observed are shown in Table 1. With the exception of benzoic acid formation from toluene, the simple oxidation reactions are due entirely to MMO activity. The formation of benzoic acid from toluene and of other acids and aldehydes shown involves MMOs plus other oxidoreductase enzymes. Dechlorination of substituted aromatic compounds and shifts in the positions of ring substituents may be manifestations of the different mechanisms of action of MMOs or may involve other enzyme activities. Debromination reactions have also been reported³⁶.

Two cases are shown in which dechlorination is associated with covalent attachment of a methyl or alkanoate group to an aromatic ring. We currently have no understanding of these reactions but it is conceivable that they are, in part, due to the lack of specificity of MMOs and they may indicate some type of free radical mechanism³⁷, or involve other enzyme activities.

In the current context, two of these examples are of special importance, namely the transformations of ethylbenzene and 1-phenylheptane. In each case, products arise as a result of side-chain oxidation and degradation. With the former compound, α -oxidation must be involved, whereas in the latter, the degradation process probably involves β -oxidation. In addition, recent experiments with 1- ^{14}C -*n*-hexadecane also suggest that *M. trichosporium* can slowly degrade long-chain alkanes; after incubation with washed cell suspensions in rigidly aseptic conditions, about 5% of the radioactivity was recovered in carbon dioxide (D.J.B., unpublished data).

Although it is well known that most methanotrophs require methane or some other reduced C_1 compounds for growth, the question arises of whether they can obtain supplementary carbon and/or energy from other sources. Clearly, ω -oxidation

of alkanes, followed by further oxidation to fatty acids which are subsequently β -oxidized will yield reducing equivalents, which might be used for energy transduction, and acetate for possible incorporation into cell material. Radiolabelling experiments have shown that products of oxidation of several gaseous alkanes and also acetate, the product of β -oxidation, are incorporated into cell material by methanotrophs³⁸⁻⁴¹. The findings of Wadzinski and Ribbons³⁹, using *Methanomonas methanooxidans*, are of particular interest. Acetate, present in the medium during growth on methane, greatly increased the growth yield and it was incorporated into cell material, especially into amino acids, as well as, in part, being oxidized to carbon dioxide by a tricarboxylic acid cycle.

Possible evolutionary reasons for the lack of specificity of methane monooxygenases

A wide range of hydrocarbons and related compounds are continually released into the environment in substantial amounts. They are natural products present in most living organisms, they arise by seepage from subterranean oil and gas deposits and are pollutants due to man's activities. There are also many hydrocarbon derivatives that are xenobiotic products of the chemical industry. Despite controversy concerning specific compounds regarded as pollutants, most hydrocarbons and hydrocarbon-like materials currently turned over in the biosphere are not novel but have been substantial components of the carbon cycle throughout most of evolutionary history. It follows, therefore, that such compounds have always been present in the environment of methanotrophs. As methane is invariably present in their natural habitats, it is not surprising that many of them have developed an obligate requirement for C_1 compounds for growth. These organisms are not in direct competition for survival with heterotrophs. They are, however, in competition with each other for available methane and it is probable that any mechanisms which might develop and enable them to utilize ancillary carbon and/or energy sources would generally be of selective value either to supplement their metabolism or facilitate survival under severe methane limitation.

Although some of the simple oxidation reactions catalysed by methanotrophs and by their MMO activities are of no obvious survival value, for example, the epoxidation of propene, others seem to be responsible for initiating useful metabolism. We would argue, therefore, that there has been selection pressure for the development of broad-specificity MMOs and other oxidoreductases. This selection pressure must be sufficient to outweigh any disadvantage of such lack of specificity. These disadvantages include truly fortuitous oxidations yielding neither energy nor products of value to these bacteria. Indeed, in these conditions, reducing equivalents would be wasted, as MMO requires a source of reducing power, which may be derived from the further oxidation of methanol, its analogues or storage polymers. In addition, some products may prove toxic. Because it is clear that these oxidations occur in the presence of

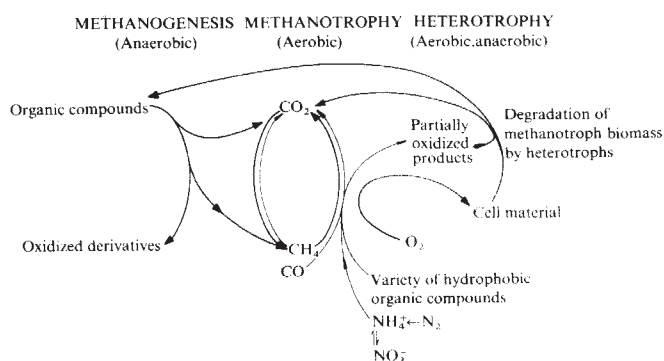


Fig. 1 Involvement of methanotrophs in the cycling of carbon and nitrogen in the biosphere.

Table 1 Examples of biotransformations effected by resting whole organism suspensions of *Methylosinus trichosporium* OB3b

Type of reaction	Compound	Products	Type of reaction	Compound	Products
Oxidation	$\text{CH}_3\text{CH}=\text{CH}_2$	$\text{CH}_3\text{CH}-\text{CH}_2$ 			$\text{O}=\text{C}-\text{C}_6\text{H}_{13}$
	Propene	Propene oxide			1-Oxo-1-phenylheptane
	Benzene	Phenol		Cinnamic acid	Benzoic acid
Oxidation and degradation	$\text{CH}_3(\text{CH}_2)_{14}\text{CH}_3$	$\text{CH}_3(\text{CH}_2)_{14}\text{CH}_2\text{OH}$	Oxidation and dechlorination		
	Hexadecane	Hexadecan-1-ol			
	Toluene	p-Hydroxytoluene			
		Benzoic acid			
	Ethyl benzene	o-Hydroxyethylbenzene			
		p-Hydroxyethylbenzene			
		2-Phenylethanol			
			Oxidation, dechlorination and condensation		
			Oxidation and condensation		
			Oxidation and rearrangement		

* Tentative identification.

methane, disadvantages may also arise through competition between methane and other potential substrates for the active site. At first sight, an equally plausible explanation for lack of specificity would not involve evolutionary arguments but would be dictated by some mechanistic requirements of MMO. However, there is substantial evidence against this view. For example, the MMO of one species, *Pseudomonas methanica*, shows greater specificity than those of other species¹⁸ and higher alkane monooxygenases that have been investigated are relatively specific. On the balance of the evidence, therefore, many of these metabolic activities are probably not fortuitous and instead have substantial metabolic significance.

It has generally been accepted that heterotrophic organisms are responsible for mineralization of hydrocarbons, their derivatives and related xenobiotics⁴². If these recently discovered, extensive metabolic activities of some species of the ubiquitous methanotrophs prove to be widespread amongst the group, it is likely that they play a significant part in the mineralization of complex, organic material, in addition to their already documented, major role in the carbon cycle. Methanotrophs are also involved in the nitrogen cycle, being able to fix atmospheric nitrogen, oxidize ammonia and reduce nitrite³.

It is clear from this discussion that methanotrophs are important in the biosphere (Fig. 1), yet only 15 years ago they

were regarded, even by most microbiologists, as obscure, uncooperative, perhaps unimportant microorganisms, as evidenced by the fact that, before 1970, only three species had been isolated and well authenticated⁷. Today, in spite of the efforts of many research groups and the isolation of some 200 strains of methanotrophs, their significance is still not widely recognized, and this fact, in part, provoked the writing of this article.

Some of the oxidative biotransformations catalysed are difficult and/or expensive to effect by conventional chemical means. The whole organism activities are relatively stable and many products are either not further degraded or are degraded only slowly. It is therefore possible that some of these species will prove valuable as industrial catalysts, exploiting intact bacteria or enzymes derived therefrom²⁰⁻²⁶.

Two important recent developments may facilitate such industrial exploitation. First, genetic manipulation of these strains is proving possible⁴³ and second, using cell-free preparations, processes may be devised in which reducing equivalents for the MMOs are supplied electrochemically, thereby dispensing with any requirement for expensive pyridine nucleotide coenzymes⁴⁴⁻⁴⁶.

We thank Mr D. Scott for helpful discussion and the SRC and ICI Limited for financial support.

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ARTICLES

Anorthositic magmas and the origin of proterozoic anorthosite massifs

Robert A. Wiebe

Department of Geology, Franklin and Marshall College, PO Box 3003, Lancaster, Pennsylvania 17604

Many anorthositic plutons of the Nain complex were formed from highly feldspathic parental magmas, which were probably generated by melting or factional crystallization of tholeiite at 15-22 kbar. Their existence suggests a high geothermal gradient and thick continental crust during the mid-Proterozoic.

PROTEROZOIC anorthosite massifs have long presented three major problems to geologists interested in crustal evolution: (1) what were the parental magmas of the anorthosite intrusions¹; (2) what is the tectonic significance of the broad belts of these intrusions; and (3) why were most of these complexes emplaced during a restricted interval of the Proterozoic²? Solution of the first problem is probably an essential precursor to solving the

other two. This article summarizes new evidence for the existence of a suite of highly feldspathic magmas parental to anorthositic rocks in Labrador.

The bulk composition of anorthosite massifs is generally much more feldspar-rich than any known magmas³. Has fractional crystallization of plagioclase from normal basaltic or andesitic magmas produced these highly feldspathic plutons, or have

these plutons been formed by unusually feldspathic magmas? If basaltic magmas were parental, complementary mafic and ultramafic intrusions must also formed. Gravity surveys indicate that many of these complexes lack hidden mafic and ultramafic bodies^{4,5}, and even where such mafic intrusions are apparently associated⁶, it has not been shown that they are comagmatic with the anorthosites. If andesitic or other intermediate magmas were parental, then complementary plutons of mafic and granitic rocks should be present. Large volumes of granite are commonly associated with anorthosites, but it is not clear that they are comagmatic with the anorthosites. Many granites seem to have been formed from separate melts generated from continental crust^{7,8}. The alternative to a 'normal' parental magma is an unusual, highly feldspathic magma. Although several works have suggested parental magmas of this type on the basis of extensive field study^{9,10}, the evidence has not been sufficient to establish a consensus that such feldspathic magmas did indeed exist and were parental to massif anorthosites. However, recent studies of three anorthositic plutons in the Nain complex of Labrador, outlined below, provide clear evidence that each of these plutons had a distinct, recognizable anorthositic parental magma. These anorthositic magmas together with high-alumina basalts^{11,12} indicate that a highly feldspathic magma series was responsible for the formation of the Nain and probably other Proterozoic anorthosite complexes.

Nain complex

The Nain complex is a typical Proterozoic anorthosite massif of area ~15,000 km² located along the coast of Labrador. It is ideal for study because it lacks later deformation and metamorphism and because contact relations are well displayed in coastal outcrops. Nearly half of the complex consists of anorthositic intrusions (colour index = 0–20); as many as 20 major anorthositic plutons may be present. Some are highly differentiated and layered; others are homogeneous and massive. Most have average plagioclase compositions between An₇₀ and An₄₀. Minor amounts of gabbroic and troctolitic rocks are present^{11,13}. Minor ferrodioritic rocks represent late stage differentiates of the anorthosites and mafic intrusions¹⁴. Somewhat less than half of the Nain complex consists of granite, most of which approaches minimum-melt composition and does not seem to be comagmatic with the anorthosites¹⁵.

Field evidence for anorthositic magmas

Two anorthositic plutons in Nain have fine-grained anorthositic margins. (Strictly, the intrusions should be termed leuconorite because they have colour indices¹⁶ in the range 10–18). One of the chilled margins represents a gradation in average plagioclase grain-size from 3 cm about 5 m in from the contact to 2 mm at the contact with country rock. The other chilled zone shows less variation and is less abrupt. These fine-grained rocks are compositionally equivalent to the coarse-grained anorthositic rocks with which they are associated. Plagioclase in all rocks is characteristically subhedral to euhedral with weak normal and oscillatory zoning. Mafic phase are orthopyroxene and inverted pigeonite with minor ilmenite, magnetite and apatite. The mineralogy suggests that the parental magma must have been relatively dry.

Fine-grained anorthositic dykes are associated with a third intrusion. They were injected into and preserved within a highly refractory xenolith of basic granulite⁸. Average grain size in many of the anorthositic dykes is 1–2 mm while the associated anorthosite has plagioclase in the range 1–7 cm.

The highly feldspathic and relatively dry magmas proposed here could exist only at high temperatures, and such temperatures are indicated by field relations along contact zones. Where anorthosite has intruded quartzfeldspathic gneisses, there is abundant evidence that the gneisses have been partially melted within 10–20 m of the contact. Granitic melt has locally mixed

with anorthositic magma or crystal mush along some contacts, and locally derived granitic veins have cut or mixed with dykes emanating from the anorthosite. Hypidiomorphic textures are common in banded gneisses near the contact. Euhedral overgrowths occur on corroded plagioclase, and interstitial areas of alkali feldspar include small euhedral crystals of quartz and biotite. In contrast to these textures, gneisses farther from the anorthosites have allotriomorphic granular textures. Berg¹⁷ has estimated that the depth of emplacement in this area was equivalent to a pressure of about 4 Kbar and that the country rock may have been only 200–300 °C before emplacement. An anhydrous magma, which was as feldspathic as the chilled rocks, must have had a liquidus temperature of ~1,300 °C. If the magma were emplaced at the temperature, the contact zone would have adjusted rapidly¹⁸ to ~800 °C, well below the solidus of the feldspathic magma and above the beginning of melting of the gneisses.

Not all contacts of anorthosites with country rock are characterized by chilled feldspathic margins and remobilized gneiss. Most other contact zones can be explained by emplacement of plagioclase-rich crystal mush with mafic interstitial liquid and by processes of crystal-liquid fractionation along the contact.

Table 1 Major and trace-element compositions and norms of proposed parental magmas of three anorthositic plutons of the Nain complex

Specimen	Kheovik Island*	Tunungayualok Island†	Uivakh Peninsula‡
SiO ₂	53.30	54.82	55.58
TiO ₂	0.28	0.94	0.77
Al ₂ O ₃	24.84	22.28	21.21
Fe ₂ O ₃	1.13	0.26	0.06
FeO	2.30	5.16	6.31
MnO	0.05	0.08	0.08
MgO	2.87	2.05	1.37
CaO	10.42	9.13	8.23
Na ₂ O	4.33	4.31	4.84
K ₂ O	0.41	0.84	1.25
P ₂ O ₅	0.07	0.13	0.30
Mg/Mg + Fe(Tot)	0.61	0.40	0.28
Norm (mol % An)	54.8	50.0	42.7
Rb	dl§	11	24
Sr	642	579	590
Ba	222	490	696
Zr	116	155	183
V	21	68	44
Ni	29	10	2
Weight norms			
Qz	—	1.68	0.46
Or	2.43	5.33	7.36
Ab + An	83.76	75.19	73.41
Di	3.32	4.76	5.53
Hy	7.33	10.56	10.98
Ol	0.83	—	—
Mag	1.63	0.38	0.09
Ilm	0.53	1.79	1.47
Ap	016	0.31	0.70

All analyses determined by X-ray fluorescence; recalculated to 100% anhydrous.

* Composition represents the average of 17 analyses of coarse-grained rocks from the interior of the pluton. This composition is very similar to that obtained for fine-grained anorthositic rocks within a chilled contact zone. These latter compositions were not used because of minor contamination by granitic melt in the contact zone.

† Composition represents the average of 17 analyses of coarse-grained rocks. This composition is very similar to that obtained for fine-grained anorthositic dykes related to this pluton⁸.

‡ Composition is based on the average of two nearly identical relatively fine-grained samples within 50 m of the intrusive contact. (REEs for two samples are listed in Table 3.)

§ Less than detection limit.

Geochemical evidence for anorthositic magmas

The chilled margins and dykes each have chemical compositions which are nearly identical to the compositions of the coarse-grained, homogeneous and massive anorthositic intrusions with which they are associated. One can thus argue that the average compositions of these intrusions must approximate their parental magmas. Estimates of the compositions of these parental magmas (Table 1) are based on the compositions of the chilled rocks or on averages of samples from the interior of the pluton. These three magmas are all highly aluminous ($Al_2O_3 = 21-25\%$) with SiO_2 between 53% and 56%. Each has a distinct Mg/Mg+Fe which varies with normative anorthite. Incompatible elements (such as, K, P, Rb, Ba and Zr) all increase as anorthite decreases. Normative colour index (wt %) varies from ~14 for the most calcic magma to ~19 for the most sodic.

Figure 1 and Table 2 present rare-earth element (REE) data for one intrusion and its associated fine-grained dykes. All analysed rocks contain approximately the same amount of plagioclase (roughly 80–90%).⁸ The remarkably constant REE content of the fine-grained dykes contrasts strongly with the variation shown by the coarse-grained rocks. The average REE content of the coarse-grained rocks approximates the REE abundance in the dykes. The similarity in REEs (as well as major and other trace elements⁹) between dykes and coarse-grained rocks strongly supports the other data which indicate that the parental magma of this intrusion was anorthositic and similar in composition to the dykes.

REEs have also been determined for rocks which approximate compositions of the parental magmas of the other two intrusions (Table 3). Two values for each pluton have been averaged and plotted on the same diagram with the average REEs of dykes related to the first pluton (Fig. 2). These REE patterns and abundances are taken to be characteristic of the magmas which have produced these three anorthositic plutons. All three have a similar light REE enrichment with a small positive Eu-anomaly. The magma with highest An (Kheovik) has the lowest REEs, and the magma with lowest An (Uivakh) has the highest REEs. There does not seem to be a systematic variation in the size of the Eu-anomaly and the Eu-anomalies are much smaller than most reported for anorthositic rocks. Although the small positive Eu-anomalies might be interpreted as being due to minor feldspar accumulation¹⁰, field relations preclude that interpretation for these rocks. Except for slight depletion in

Table 2 REEs in rocks related to the Tunungayualok Island intrusion

Spec	Dykes				Coarse-grained rocks			
	500B*	715B*	718A*	500D†	122A†	218†	509†	CS546*
La				14.9	20.8	18.2	4.44	
Ce	31.0	26.1	32.7	29.5	44.9	39.3	8.24	19.8
Nd	14.0	13.2	15.1	12.6	20.0	19.0	3.95	10.6
Sm	2.60	2.55	2.76	2.54	4.10	3.86	0.788	2.14
Eu	1.57	1.54	1.41	1.54	1.54	1.51	1.09	1.53
Gd	2.32	2.33	2.45					2.07
Tb				0.35	0.64	0.67	0.146	
Dy	1.97	2.05	2.03					1.88
Er	1.04	1.12	1.06					1.07
Yb	0.922	1.03	0.920	0.955	1.98	1.58	0.445	0.985
Lu				0.153	0.299	0.258	0.066	0.147

* Isotope dilution analyses by E. C. Simmons

† Instrumental neutron activation analysis (INAA) by R.A.W.

heavy REEs, the REE abundances of these anorthositic rocks fall within the range of values for Hawaiian basalts²⁰. In terms of REEs and major elements these anorthositic rocks closely resemble anorthositic parental magmas estimated by Simmons and Hanson²¹.

Origin of anorthositic magmas

Highly feldspathic magmas provide an immediate explanation for the existence of the Proterozoic anorthositic massifs. However, the source and genesis of these magmas remain significant problems. The fact that An and Mg/Mg+Fe vary sympathetically suggests that these magmas were in equilibrium with both plagioclase and pyroxene and that they therefore represent cotectic compositions. Available experimental data suggest that an appropriate cotectic with at least 75–80% plagioclase is present at pressures in the range 15–22 kbar in olivine-free tholeiitic compositions^{22,23}. If they represent partial melts, their highly feldspathic compositions suggest that feldspar was in the source area and that a high proportion of the feldspar was melted. Light REE enrichment and small positive Eu-anomalies also suggest major melting of feldspar²¹. A pure feldspar source (for example, Archean anorthosite) is extremely unlikely because of the sympathetic variation of Mg/Mg+Fe with normative An in the three magmas. Direct derivation by partial melting of the mantle seems unlikely because of the low Mg/Mg+Fe (ref. 21).

Alternatively, the feldspathic magmas reported here may have been derived by fractional crystallization from tholeiitic magma²⁴. Possible distribution coefficients for REEs²¹ indicate that fractional crystallization of clinopyroxene could lead to a magma enriched in light REEs and depleted in heavy REEs, and develop a positive Eu anomaly from a liquid initially lacking one. Cotectic crystallization of plagioclase would also increase REEs and could be responsible for the smaller Eu of the Uivakh magma. Proterozoic dykes which cut the Nain complex have recently been found to carry megacrysts of high-Al orthopyroxene and cumulate nodules of plagioclase and two

Table 3 REEs in rocks of the Kheovik Island and Uivakh Peninsula intrusions

Spec	Kheovik Island		Uivakh Peninsula	
	777	779	856	857
La	9.03	10.17	23.54	23.09
Ce	19.96	21.40	51.43	50.74
Nd	8.9	11.1	32.1	28.2
Sm	1.86	1.72	5.14	5.44
Eu	0.88	0.92	2.12	2.19
Tb	0.226	0.206	0.732	0.835
Yb	0.550	0.644	1.71	2.03
Lu	0.064	0.100	0.254	0.318

All analyses were determined by INAA at the NASA Johnson Space Center in Houston.

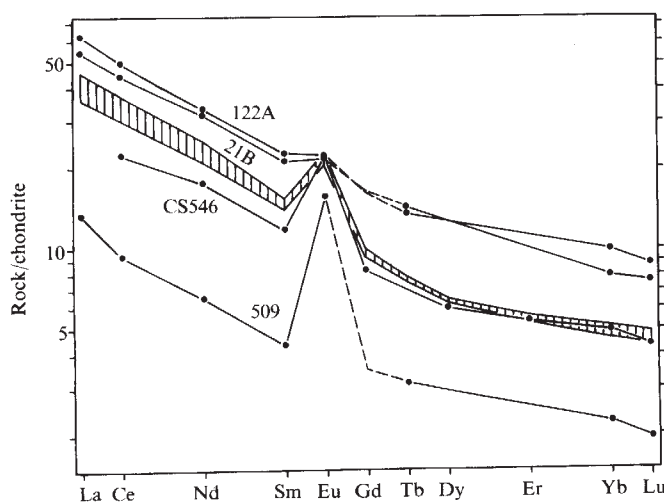


Fig. 1 Chondrite-normalized REEs from coarse-grained anorthositic rocks and fine-grained dykes related to the Tunungayualok Island intrusion. Analyses are listed in Table 2. Vertically ruled pattern shows the complete range of REEs in the fine-grained dykes.

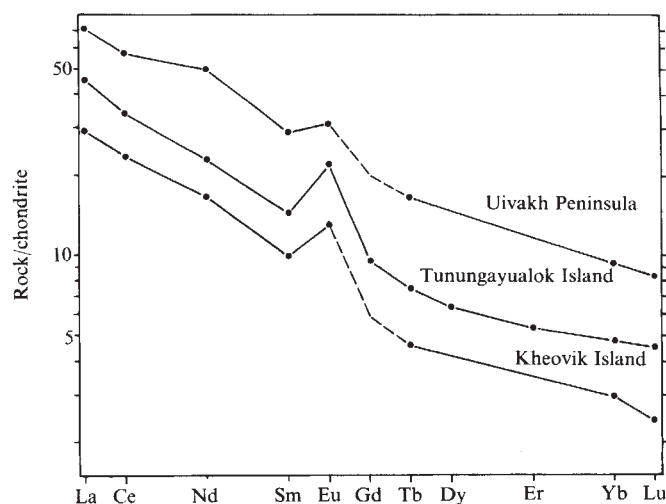


Fig. 2 Chondrite-normalized REEs of proposed parental magmas for three anorthositic plutons of the Nain complex. Values for the Kheovik Island and Uivakh Peninsula plutons are averages of analyses in Table 3. Values for the Tunungayualok Island intrusion are averages of the four analysed fine-grained dykes.

pyroxenes. The composition of these minerals suggest that they would represent lower crustal cumulates responsible for the fractionation from basaltic to anorthositic magma.

If these magmas have been generated from tholeiitic compositions, the need for a feldspar-rich cotectic suggests a tectonic environment characterized by a relatively high geothermal gradient and thick crust. An intracontinental rift zone might provide conditions necessary to generate these anor-

thositic magmas, and recent workers have argued that the Proterozoic anorthosite massifs have developed in such an environment^{2,17,24}.

Conclusions

The anorthositic magmas proposed here are documented by chilled margins and fine-grained dykes. They have compositions essentially identical to the coarse-grained, massive, homogeneous anorthositic intrusions with which they are associated. Although only three different magmas have been documented so far in Nain, it seems highly probable the many other similar but distinct plutons of massive anorthositic rocks will prove to have crystallized from other batches of feldspathic magma. There is also abundant evidence that high Al basaltic magmas make volumetrically significant contributions to the anorthosite complexes^{11,12,24}.

The anorthositic rocks within the Proterozoic complexes, therefore, seem to have been generated by magmas ranging in composition from high-Al basalt^{11,24} to the highly feldspathic magmas reported here. These magmas define an entirely new association which is clearly distinct from previously known basaltic and calc-alkaline series. This unique magma association implies that unique tectonic and thermal settings are responsible for the generation of the Proterozoic anorthosite massifs. If most of these massifs prove to have ages in a restricted time period (for example 1,400–1,500 Myr), as present evidence suggests²⁴, then the conditions necessary to generate that magma series must have been most common in that period. Recognition that unusually feldspathic magmas are involved in anorthosite genesis should aid in evaluating how tectonic style, crustal thickness, and the geothermal gradient evolved in Proterozoic time.

This work was supported by NSF grants EAR 73-00667 A02 and EAR 75-14423 A01.

Received 21 February; accepted 5 June 1980.

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High-salt d(CpGpCpG), a left-handed Z' DNA double helix

Horace Drew, Tsunehiro Takano, Shoji Tanaka, Keiichi Itakura* & Richard E. Dickerson

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

*City of Hope National Medical Center, Duarte, California 91010

The DNA tetramer d(CpGpCpG) or CGCG crystallizes from high-salt solution as a left-handed double helix, the Z' helix. Its structure differs from that of the other known left-handed helix, Z-DNA, by a C1'-exo sugar pucker at deoxyguanosines rather than C3'-endo, and these represent two alternative solutions to the same steric constraint arising from the syn glycosyl bond orientation. The apparent molecular basis for the Z to Z' transition in going from intermediate to high salt is substitution of a bound anion for water at guanine amino groups, and consequent charge repulsion of anions and backbone phosphates.

THE predominance of right-handed DNA helices of the A and B families was called into question recently by the discovery¹ that the double-helical DNA hexamer d(CpGpCpGpCpG) is a left-handed helix, and one in which the repeating unit is two base pairs rather than a single step. This 'Z helix' has since been found to explain the X-ray patterns from certain fibres with alternating

purine-pyrimidine sequences². We now report that the high-salt form of the tetramer d(CpGpCpG) (hereafter to be termed CGCG) also adopts a left-handed conformation, similar to but not identical with the Z helix.

We, like Wang¹, chose alternating CG oligomers of DNA for study because it has been known for some time^{3–6} that

alternating poly (dC-dG) undergoes a reversible, salt-induced conformation change with a midpoint at about 0.7 M MgCl_2 or 2.5 M NaCl (essentially the same ionic strengths); the exact nature of this change has been a matter of interest. We have reported that such a transition with a change in ionic strength of the surrounding medium can also be observed with the tetramer CGCG in the crystalline state itself⁷. Hexagonal crystals of a low-salt form can be grown by diffusion of ethanol into aqueous solutions of CGCG (for experimental details as well as crystallographic survey data, see ref. 7). High-salt crystals are grown by vapour diffusion from solutions containing DNA, MgCl_2 and 2-methyl-2,4-pentanediol (MPD). The hexagonal low-salt crystals that form initially in this latter method crack into smaller blocks as the MgCl_2 concentration approaches 1 M. These blocks and the crystals that nucleate subsequently show the orthorhombic X-ray pattern of the high-salt form. Conversely, high-salt crystals that are placed in a more dilute solution convert immediately from the orthorhombic to a slightly disordered version of the hexagonal X-ray pattern⁷. Although the diffraction intensity distribution is quite different in the two forms, the transition is apparently small enough to be accommodated within the crystal lattice packing. Unit cell dimensions suggest that the transition involves a shifting of the double helices relative to one another along the c axis, with the lateral packing of helices being relatively unaffected, as in Fig. 3 of ref. 7. The structure of the high-salt form is now known and is the subject of the present article.

Structure analysis

Crystallization data and preliminary X-ray survey results have already been reported⁷. High-salt crystals are orthorhombic, space group $C222_1$, with cell dimensions $a = 19.50 \text{ \AA}$, $b = 31.27 \text{ \AA}$, $c = 64.67 \text{ \AA}$, and with one CGCG double helix (two CGCG monomer strands) per crystallographic asymmetric unit. The empirical formula of $\text{C}_{38}\text{N}_{16}\text{O}_{22}\text{H}_{46}\text{P}_3$ per strand leads to a molecular weight of 1,172, and the measured crystal density of 1.6 g cm^{-3} indicates that the crystals are only 49% DNA by

weight. The structure was solved by the method of single isomorphous replacement. Diffusion of various ions and complexes into pre-grown CGCG crystals failed to produce a usable isomorphous derivative. Instead, two covalent heavy atom derivatives were prepared by *de novo* synthesis of the tetramer, with 5-bromocytosine in place of one of the cytosines at the first or third position, using the triester synthesis⁸. The derivative with bromine at the third position (3Br-CGCG) crystallized isomorphously with the native compound, whereas that with bromine at the first position (1Br-CGCG) did not. In the light of the final structure, we now can see that insertion of bromine at the beginning of the molecule interferes with stacking of adjacent double helices up the c axis, whereas 3Br-CGCG has no such impediment to stacking. Single isomorphous replacement phases⁹ calculated from the 3Br derivative yielded a mean figure of merit of 0.42 to a resolution of $2.7 \text{ \AA}$. These phases were used to calculate a 'best' or least r.m.s. error electron density map¹⁰. A Kendrew wire model served as the starting point for Jack-Levitt refinement¹¹. The completed structure consists of 158 non-hydrogen DNA atoms and 86 ordered solvent atoms per asymmetric unit. The current residual error or R factor for 1,900 reflections ($>2\sigma$) to a resolution of $1.5 \text{ \AA}$ (beyond which the pattern fades rapidly) is 19.9%.

The CGCG double helix

As shown in Fig. 1, two CGCG strands in the high-salt crystals form a short left-handed antiparallel double helix with Watson-Crick base pairing. As in the Z helix reported for the CGCGCG hexamer¹, cytosines retain their customary *anti* orientation about the C-N glycosyl bond, with the sugar ring turned away from the O2 carbonyl oxygen of the cytosine ring, and guanines adopt an unusual *syn* conformation with the sugar ring bent towards the guanine N3 ring nitrogen. The sugar-phosphate backbone therefore has a zigzag path, with two steps along the chain as the true helical repeat. The helical rotation between repeating units or pairs of base pair steps is -60° . The minor

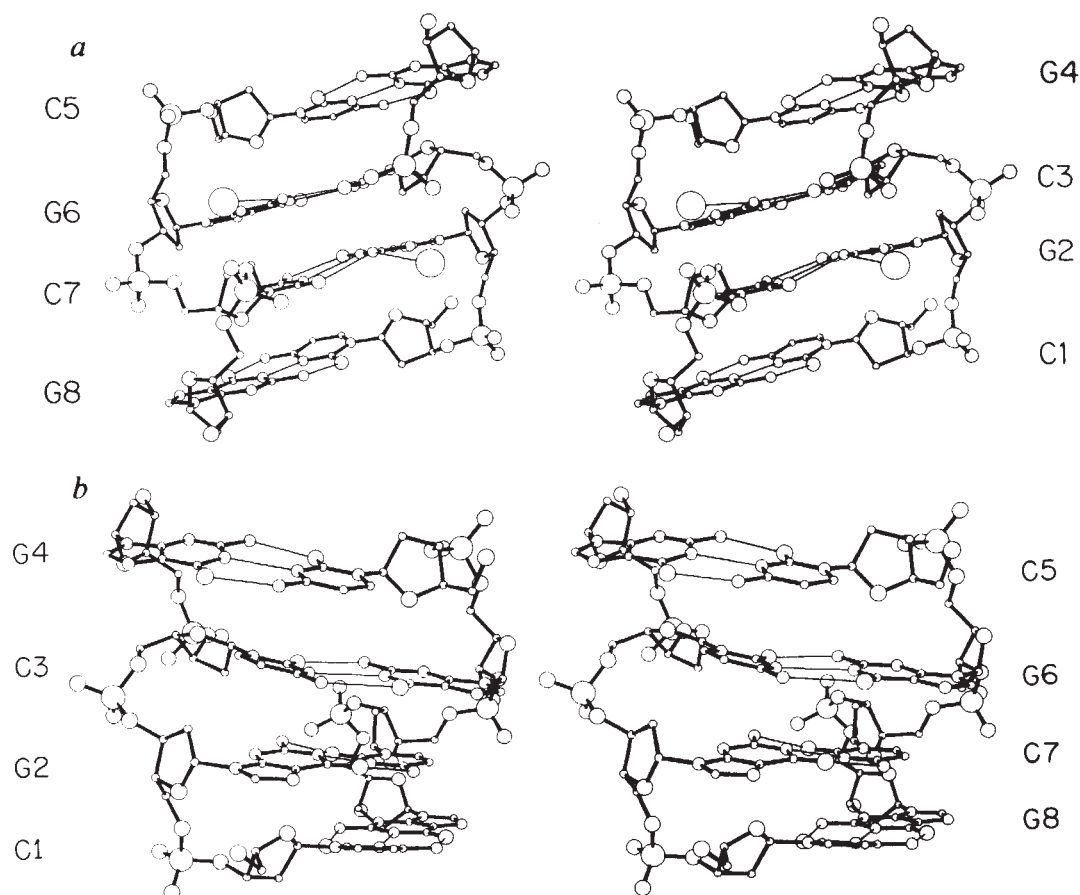


Fig. 1 Stereo pair drawings of the high-salt CGCG double helix. Cytosine and guanine bases are labelled C1 to G8 to the left and right of the two stereo images. Two chloride ions are shown in *a* as large spheres coordinated to the N2 amides of guanines G2 and G6. Other spheres in order of decreasing radius represent P, O, N and C. The phosphorus atoms along the backbone are referred to as P2, P3 and P4 in one strand and P6, P7 and P8 in the other. *a*, View into the minor groove, down the approximate 2-fold axis. *b*, Oblique view from the outside, in the direction that would correspond to the major groove in the right-handed helices.

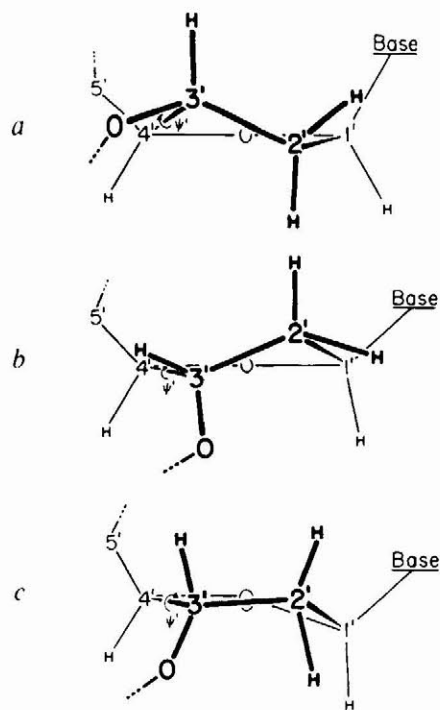


Fig. 2 Deoxyribose ring conformations: a, C3'-endo; b, C2'-endo; c, C1'-exo. The value of the C4'-C3' torsion angle ψ' is clearly defined by the positions of atoms O3' (attached to C3') and C5', and is 82° (a), 144° (b), and 120° (c) for the conformations drawn here.

groove containing the guanine amino and cytosine carbonyl groups (Fig. 1a) is the only true groove in the helix, because what corresponds formally to the major groove with guanine carbonyl and cytosine amino groups (Fig. 1b) is pushed out to the surface of the helix so that it is not a groove at all. The *syn-anti* alternation of glycosyl bonds may limit all such zigzag left-handed helices to alternating purine-pyrimidine sequences, because placing a pyrimidine base in the *syn* conformation brings its O2 carbonyl oxygen close enough to the O1' oxygen of deoxyribose to disturb their first hydration shells and permit dipole-dipole repulsion.

The low-salt CGCGCG hexamer¹ and the present high-salt CGCG tetramer differ slightly in the tilt of base planes to the helix axis and base repeat spacing along the helix axis. In the hexamer the bases are tilted 7° away from perpendicularity to the helix axis and the average rise per residue along the axis is 3.7 Å. In the tetramer the base plane tilt is 9° and the average rise per residue is 3.8 Å. These differences are associated with a more fundamental change in sugar conformation at deoxyguanosine. In the hexamer the sugar pucker is C2'-endo at cytosines and C3'-endo at guanines. The tetramer also has C2'-endo sugars at cytosines, but at guanines the conformation is C1'-exo. This latter is a conformational variant of C2'-endo, and quite distinct from C3'-endo (Fig. 2). Hence, to a crude first approximation, the sugar pucker is repetitive along the CGCG chain rather than alternating as in CGCGCG.

The evidence for sugar pucker comes less from direct observation of the rings themselves than from the geometry of the backbone chain. As the X-ray data for the tetramer do not extend to atomic resolution, every atom in the deoxyribose ring cannot be resolved and positioned accurately from electron density alone. A better guide to sugar pucker is the torsional angle about the C4'-C3' bond in the five-membered ring (ψ' in Fig. 2). The X-ray results allow this angle to be specified with considerable accuracy, as the C5'-C4'-C3'-O3' linkage is an extended chain with well resolved atoms. This torsional angle is directly related to the sugar pucker as shown in Fig. 2: ~82° for the C3'-endo configuration, 144° for C2'-endo and an intermediate 120° for C1'-exo (ref. 12). In the CGCG structure it

averages $141 \pm 4^\circ$ for the four cytosine positions, and $122 \pm 1^\circ$ for three of the four guanines; at one of the 3'-terminal guanines, G8, the final O3' oxygen is disordered, so its ψ' value of 141° is not representative of the ideal helical structure.

The C1'-exo conformation can be regarded as a slightly 'bent' C2'-endo, and this deformation can be seen from the tetramer structure to arise from a close contact between the N3 nitrogen atom of a guanine ring and the C2' atom in the sugar ring that is in *syn* orientation to it. This distance averages 3.1 ± 0.1 Å in the crystal, with a C1'-exo pucker. Forcing the ring back into a C2'-endo conformation would push these atoms 0.4 Å closer together, leading to an unacceptably short non-bonded contact. The observed tetramer backbone, therefore, represents the best that can be done using a repetitive C2'-endo-like sugar pucker, in the presence of alternating *syn* and *anti* glycosyl bonds. The alternation of 2'-endo and 3'-endo present in the CGCGCG hexamer¹ represents a different solution to the same steric problem.

We have termed our structure the Z' helix because it differs from the Z helix to about the same degree, if in a different way, as the A' helix with 12 base pairs per turn^{13,14} does from the A helix with 11 (ref. 15). Transitions between both pairs, A to A' and Z to Z', can be induced by changes in salt concentration. A similar salt-induced transition is observed between the two principal members of the third family of helices, B with 10 base pairs per turn and C with 9½ (refs 16-18). From this viewpoint it might have been more consistent for the C helix to be termed the B', but consistency in an evolving scientific nomenclature is a goal scarcely to be hoped for.

Unlike CGCGCG, the molecular helix axis in CGCG does not coincide with a crystallographic axis, and the molecules are not stacked in such a way as to simulate a continuous helix. In Fig. 3, the two molecules are related by a 2-fold axis normal to the plane of the paper through the centre of the diagram. Horizontal 2-fold axes above and below these molecules also relate them to other molecules not shown at the top and bottom of Fig. 3. An elongated solvent peak of eight electrons occupancy extends between positions 3.5 Å away from the N2 amino groups of guanines G4 on the two molecules shown, and the distance from the amino group suggests that this is a partial-occupancy, disordered chloride site rather than water. At the other end of each molecule, the amino group of guanine G8 is coordinated to a water molecule at 2.7 Å distance rather than to C1', and a network of solvent molecules crosses each horizontal 2-fold axis to connect to another molecule not shown. Each molecular helix

Table 1 Comparison of left-handed DNA helices

Helix	Z'	Z
Where found	High-salt CGCG	Low-salt CGCGCG
Repeating units per turn	6.07 ± 0.31	6
Lateral crystal packing diameter*	18.4 Å	18.1 Å
Rise per repeat	7.61 ± 0.11 Å	7.43 Å†
Base pair tilt to helix axis	9°	7°
Rotation per repeating unit	$-59.4 \pm 3.1^\circ$	-60°
Glycosyl torsion angle (χ)		
Deoxycytidine	<i>anti</i> (23°)	<i>anti</i> (21°, 32°)‡
Deoxyguanosine	<i>syn</i> (-106°)	<i>syn</i> (-112°, -118°)‡
Sugar pucker (ψ')		
Deoxycytidine	C2'-endo (141°)	C2'-endo (138°, 147°)‡
Deoxyguanosine	C1'-exo (122°)	C3'-endo§ (99°, 94°)‡
Distance of P from axis		
d(CpG)	6.0 ± 0.3 Å	6.9 Å
d(GpC)	8.2 ± 0.4 Å	8.0 Å
P-P distance across helix		
d(CpG)	11.4 and 12.1 Å	12.5 Å
d(GpC)	16.5 Å	15 Å
Ref.	This work	1

As the repeating unit in each helix is two consecutive bases, C and parameters given have been expressed in terms of this repeat, individual bases. Idealized parameters for the Z' helix have been obtained from a helix-building program provided by John Rosenberg.

* $d = 0.5(a^2 + b^2)^{1/2}$, where a and b are crystal cell dimensions.

† $c/6$, where c is the cell dimension along the helix axis.

‡Variants Z_I and Z_{II}, respectively.

§Reported as C3'-endo, but with torsion angles closer to C2'-e

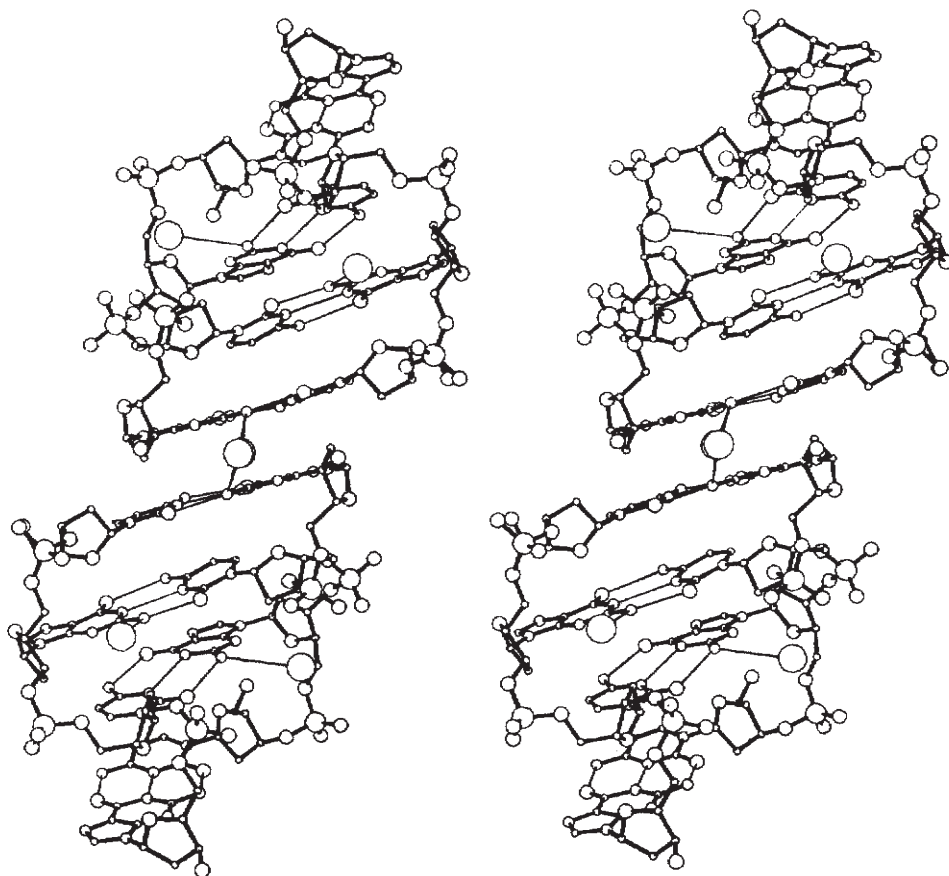


Fig. 3 Stereo drawing of stacking of neighbouring molecules along the *c* axis (vertical). These two molecules are related by a crystallographic 2-fold axis normal to the plane of the drawing through the central bridging chloride ion (large circle). This ion is actually a cigar-shaped peak elongated vertically from one guanine plane to the other, and could represent statistical occupancy of two sterically exclusive coordination positions to the two G4 guanine rings.

axis is tilted 14.3° away from the crystallographic *c* axis, which is vertical in Fig. 3.

In addition to this mismatch of molecular helix axes, the two molecules shown are not stacked in a way that continues the helical twist. For this to be true, the lower two base pairs of the top molecule in Fig. 3 should be rotated by -60° relative to the upper two base pairs of the bottom molecule. The observed rotation is only -30° , and the reason for this smaller rotation is that it maximizes base pair overlap between molecules. The absence of linking phosphate groups permits this more favourable stacking with base pairs almost eclipsing one another, whereas within a helix the GpC base stacking involves a 32° twist. The combination of a 60° helical twist within each molecule and a 30° non-helical twist between molecules turns the *c* axis into a pseudo- 4_3 screw axis, leading to a strengthening of $(0, 0, l)$ reflections with $l = 4n$ and weakening of the others, as was noted from the first survey X-ray photographs⁷. The molecular packing is exactly as was predicted in Fig. 7 of ref. 7, except that the lateral 2-fold axes run between molecules rather than through the molecular centres. The 2-fold symmetry of the two halves of any one molecule is only approximate.

The idealized Z' helix

To establish the helix axis and other helical parameters as shown in Table 1, the rotational and translational parameters that best mapped the first CG half of the molecule onto the second half had to be found analytically. As with CGCGCG, there are six repeating units per turn of ideal continuous helix. The rise per repeat and the tilt of base planes to the helix axis are both slightly greater in CGCG than in CGCGCG, but the most conspicuous differences are the deoxyguanosine sugar puckering, mentioned earlier, and the phosphate separation distances listed in Table 1.

Figure 4 shows three CGCG molecules stacked on top of one another with their helix axes coincident and with -60° rotation between them to simulate one complete turn of Z' helix. Although each molecule is correct as observed, this idealized arrangement of them has no relationship to the actual molecular

packing in the crystals. Zigzag lines connect the phosphorus atoms, including missing phosphates necessary to turn the three molecules into a continuous helix. As was noted for the Z helix, each cytosine deoxyribose ring has its oxygen atom packed against a guanine ring one step farther along the chain. Each base plane in Fig. 4 is puckered or twisted, and the observed deformations imply a steric repulsion by this close-packed sugar oxygen. A careful examination of Fig. 2 of ref. 1 reveals a similar apparent repulsion between guanine rings and deoxyribose oxygens in the Z helix, and this may be a characteristic feature of these left-handed zigzag structures.

Ion binding and helix conformational change

CGCGCG was crystallized from low-salt conditions and CGCG from high salt. The explanation for their observed difference in backbone conformation, and also for the salt dependence of the transition, may lie in the presence of bound chloride ions in the CGCG structure. DNA itself is a polyanion; if one assumes a charge of -3 on each CGCG strand and the observed crystal packing, then the crystals are 2.0 M in negative charges which must be neutralized by cations. By comparison, crystals of CGCGCG with an assumed -5 charge per strand and the published cell constants¹ are 2.6 M in negative charge, which sets a lower limit on the compensating cationic charge molarity.

Table 2 Solvent binding to base amino groups in CGCG

Base no.	N-to-solvent distance (Å)	Refined total electrons	Probable identity
Minor groove:	N2 of guanines		
G2	3.6	16	Cl ⁻
G4	3.5	8	Cl ⁻
G6	3.2	11	Cl ⁻
G8	2.7	5	H ₂ O
Major groove:	N4 of cytosines		
C1	—	—	—
C3	3.1	4	H ₂ O or Cl ⁻ ?
C5	3.2	7	Cl ⁻
C7	3.6	8	Cl ⁻

Although CGCGCG was crystallized from low-salt solution conditions, it is by no means in a low-salt or low-ionic strength environment within the crystals themselves, and this may be significant for the helix conformation in the crystal. CGCGCG might better be described as an intermediate salt structure.

As mentioned earlier, high-salt CGCG has additional chloride anions bound within the crystal structure, and these are listed in Table 2. In the minor groove, two sites of occupancy 16 and 11 electrons are found 3.6 Å and 3.2 Å away from the N2 amino groups of guanines G2 and G6 (Fig. 1a). An elongated 8-electron peak bridges N2 amino groups of guanines G4 on adjacent molecules as depicted in Fig. 3. Two more partial-occupancy chloride sites are coordinated to amino group N4 of cytosines C5 and C7 on the 'major groove' surface of the molecule. Hence, allowing for partial occupancy and other possible minor sites, we estimate that there are approximately four bound chlorides per double-stranded helix or two per monomer. This leads to a total negative charge molarity from DNA plus chloride of 3.7 M, which again places a lower limit on compensating cation molarity.

The phosphate group connecting bases G6 and C7 in CGCG is rotated out and away from the narrow groove relative to its position in CGCGCG, in a manner which suggests electrostatic repulsion between it and the chloride bound to G6 (Fig. 1a). The phosphate connecting G2 and C3 is rotated away from the chloride bound to G2 in a similar manner, and together these account for the 1.5 Å increase in GpC phosphate separation in the Z' helix shown in Table 1. By contrast, in CGCGCG the phosphate and the guanine N2 amino groups are actually

bridged by a hydrogen-bonding water molecule, as shown schematically in the left half of Fig. 5. This difference in phosphate position is sufficient to account for the increase in torsion angle ψ' from 82° in CGCGCG to 122° in CGCG, and the change in sugar conformation at guanine from C3'-endo to C1'-exo. It is significant that Pohl and Jovin³ did not see a salt-induced conformational change in either poly(dI-dC) or poly(dA-dT), both of which lack the N2 amino group in the minor groove. Patel's observation that poly(dI-dC) shows only a single phosphorus NMR resonance¹⁹ rather than the two found with poly(dG-dC)²⁰ indicates that the (dI-dC) polymer is not already in a zigzag left-handed helix in the conditions examined. It may be that the loss of the solvent interaction in Fig. 5 tips a delicate free energy balance in favour of a right-handed helix.

This is the first single-crystal study of an alteration in double-helix geometry produced by binding of ions, but not the first time that such mechanisms have been invoked to explain transitions monitored by circular dichroism or other methods. Both the A to A' transition^{13,14} and the B to C¹⁶⁻¹⁹ may involve specific ion binding, and there may be a certain amount of parallelism in the behaviour of the three principal families of nucleic acid double helices: A, B and Z.

Why a left-handed helix at all?

To date, X-ray, circular dichroism, laser raman and/or NMR evidence has been found for a salt- or alcohol-induced transition that probably involves a right- to left-handed helix change, with poly(dG-dC) (ref. 3) and poly(dI-Br⁵dC) (ref. 20), but not with

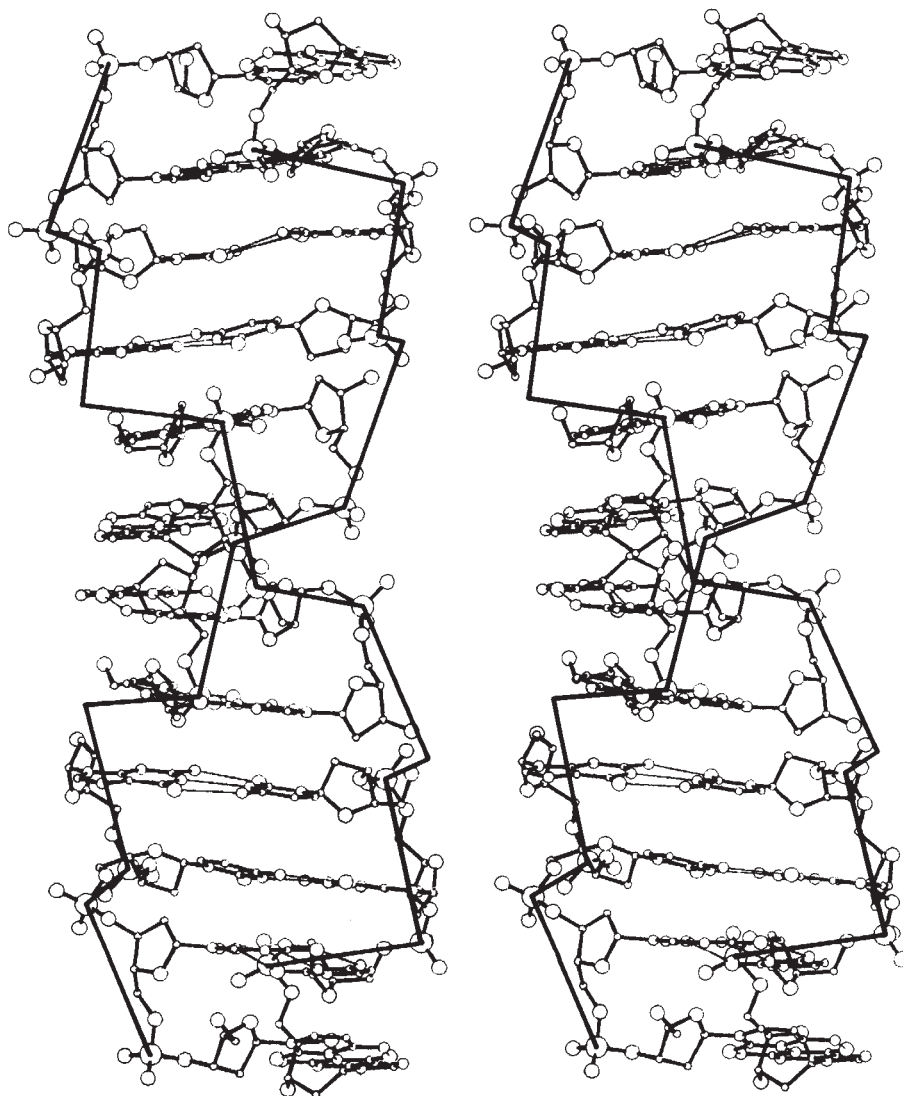


Fig. 4 Stereo view of stacking of three CGCG molecules on top of one another in such a way as to simulate a continuous 6-fold Z' helix. Ziggzag lines connect the phosphate backbone of an ideal helix, including the absent phosphates between molecules. The base plane normal is tilted 9° to the helix axis. The nearest phosphates on opposite strands are only 8.1 Å apart across the minor groove. The 'major groove' is pushed to the outside of the helix and is not a groove at all. Base plane puckering is such as to suggest steric repulsion by adjacent cytosine deoxyribose oxygen atoms. This is an isometric drawing with the viewing point at infinity; previous stereo figures have been perspective drawings with a finite viewing distance.

Table 3 Nucleic acid double-helix geometry

Family	A		B		Z	
Helix sense	Right		Right		Left	
Glycosyl bonds	<i>anti</i>		<i>anti</i>		<i>syn/anti</i>	
Sugar pucker	C3'- <i>endo</i>		C3'- <i>exo</i> (or <i>endo</i>)		Alternating	
Helix	A	A'	B	C	Z	Z'
Bases per turn	11	12	10.4–10*	9 $\frac{1}{2}$	12	12
Rotation per base	32.7°	30°	34.6°–36°	38.6°	–60°/2	–60°/2
Pitch (Å)	30.9	36	33.8	31	44.6	45.7
Rise per base (Å)	2.8	3.0	3.38	3.31	7.43/2	7.61/2
Lateral packing diameter (Å)	23	23	19.3	19.2	18.1	18.4
Base tilt	16°–19°	10°	–6°	–8°	–7°	–9°
Refs						
Conformation	15	13, 14	15	16, 18	1	This work
Transformations within family	13, 14, 17		16–18, 29, 30		7, this work	
Transformation from B family	16, 21, 28		—		3–7, 20	

*Fibre diffraction experiments indicate 10 base pairs per turn, but solution measurements³¹ and theoretical calculations³² favour a slightly larger non-integral value. The entire B–C family probably represents a continuum of rotation angles in the range 34°–39°.

poly(dI-dC) itself, poly(dA-dT), poly(dA-Br⁵dU), poly(dA-I⁵dU), the ribonucleic acid poly(rG-rC), or any sequence lacking a purine/pyrimidine alternation^{3,20}. It is not difficult to see why zigzag left-handed helices might be limited to such alternating sequences: the steric repulsion mentioned earlier between cytosine and a *syn* deoxyribose ring may be sufficient. However, why should even poly(dG-dC) adopt a left-handed geometry in preference to a right-handed helix of the A or B families, and why the apparent discrimination between various possible alternating sequences?

Properties of the six principal helices in the three helix families are compared in Table 3, and their known interconversions are schematized in Fig. 6. The three sectors of the circle about the B helix represent the three ways of bringing about transitions: lowering the humidity of fibres or films, increasing the alcohol concentration, or raising the ionic strength by increasing the salt concentration. Transitions within one family seem to be salt dependent and may even involve specific ion binding. This is certainly true for anion binding in the Z to Z' transition, as the X-ray results have demonstrated. Ivanov¹⁷ has also observed a correlation between the relative abilities of alkali metal cations to induce the B to C transition, the radii of the hydrated cations and the width of the minor groove as the helix winds down from 10 base pairs per turn to 9 $\frac{1}{2}$. Little

information is available about the A to A' transition in DNA, but in RNA the transition is salt induced. All these interconversions within families are probably non-cooperative as they occur gradually over a reasonably broad range of conditions.

The transitions between the B helix and A or Z families are sharper and are cooperative in nature^{4,21}. This is not surprising, because a major change in backbone conformation at one point along the helix will make it easier for neighbouring regions to adopt the same changes, and for the transformation to propagate along the helix. Only the B to C conversion is appreciably temperature sensitive: with a ΔH° of –10 kcal, the C form is increasingly favoured as the temperature falls^{22,23}. In contrast, the B to A and B to Z transitions are essentially isenthalpic and independent of temperature^{3,21}.

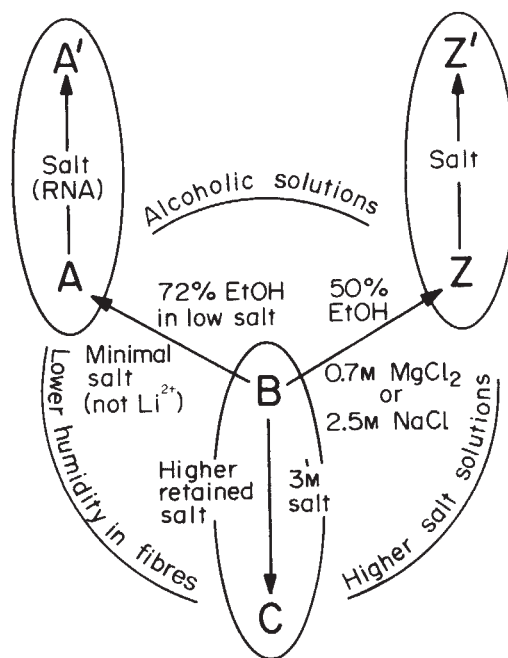


Fig. 6 Families of double helices and transitions between them. Transitions between members of one family, within oval outlines, are influenced by salt concentration. Transitions between families can be brought about by changing relative humidity and salt content in fibres or films, or changing ionic strength or solvent polarity in solution. Critical salt and ethanol concentrations, from refs. 3, 6, 17, are expressed as midpoints rather than endpoints of transitions. The B to Z transition, involving a change in helix sense, may be restricted only to certain alternating purine/pyrimidine sequences.

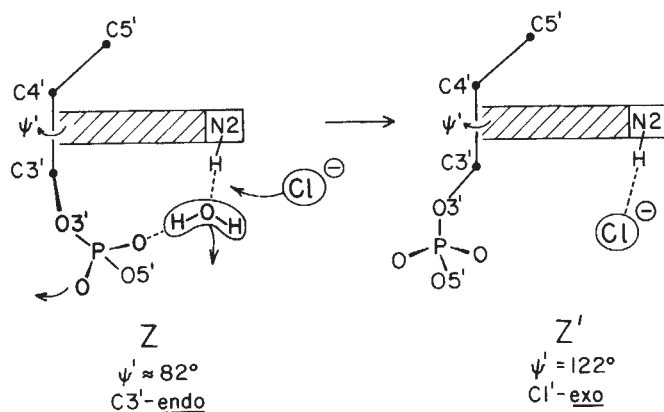


Fig. 5 Interactions between deoxyguanosine O3' phosphate and N2 amino group in the Z helix of low-salt CGCGCG (left) and Z' helix of high-salt CGCG (right). In CGCGCG the two groups are bridged by a water molecule, whereas in CGCG the phosphate is repelled by a bound chloride. This can account for the observed increase in ψ' (the C4'–C3' torsion angle) from 82° in CGCGCG to 122° in CGCG, and the change in sugar pucker from C3'-*endo* to C1'-*exo*.

Ions in high-salt conditions could be imagined to have any of three roles in effecting helix transformations: (1) specific binding to DNA, (2) charge-cloud screening of phosphate-phosphate backbone repulsions, or (3) a general lowering of activity of water molecules because of hydration of ions. Phosphate screening has been invoked to explain both the B to Z and the B to C transitions^{1,3,17}, but this seems unlikely for two reasons. Although phosphate groups on different helix strands are closer together in the Z or C helices than in the B, they are fully as close in A and Z. If the screening argument were valid, the A helix should be stabilized by high-salt conditions. In fact, it seems to be the form of choice only in minimal salt conditions, whether in fibres or in solution. Moreover, the B to Z conversion is favoured both by alcohol and by salt, and these have opposite effects on phosphate repulsions: salt diminishes the repulsion by charge-cloud screening, but alcohol increases the strength of negative charge repulsion by lowering the dielectric constant of the surrounding medium. Manning²⁴ has also pointed out that, because of the phenomenon of charge condensation along a polyelectrolyte, the local ion concentration around a polyelectrolyte charge and its degree of neutralization do not follow a simple law of mass action; the phosphate charges in DNA may be largely neutralized by even a small excess of cations, as recent ²³Na NMR experiments have shown²⁵.

The one effect that is shared by the drying of fibres, increasing salt concentration and increasing the amounts of such solvents as ethanol, isopropanol or dioxane, is a general lowering of the activity or effective concentration of water molecules. Each treatment, in a sense, is a kind of local dehydration of the polymer. If one regards the B form as most stabilized by interactions with water molecules, whether specific or nonspecific, and the A and Z forms as less involved with water, then all of the inter-family transitions in Fig. 6 become consistent. Although achieved by different means, they have a common result. This interpretation would also explain why both the 'low-salt' hexamer and the high-salt tetramer of CG adopt a Z conformation in the crystal, because the water activity is lowered by both the high DNA concentration and the high cation concentration required for charge neutrality. The act of crystallization is a process of concentration for both DNA and ions.

As Fig. 6 suggests, the left-handed Z helix family actually seems to be the conformation of choice when water activity is lowered, but only if the base sequence permits its adoption. At present, this seems to require an alternating purine/pyrimidine sequence, or even poly(dG-dC). Is this preference for the Z helix

sufficiently strong to produce it even if adjacent regions of DNA are of sequences that cannot adopt the Z conformation? More information is needed from crystal structure analyses of related double helices. Data have been collected in this laboratory by Drew and Wing²⁶ on a self-complementary DNA dodecamer with the sequence CGCGAATTCGCG, having adjacent Z-compatible and Z-incompatible segments. Other dodecamers are being synthesized with sequences that, hopefully, will exhibit classical B or C helix structures. These, and other carefully chosen sequence variants, should greatly assist the unravelling of the importance of solvent and other factors in stabilizing different helical types, the significance of the new left-handed structures and their ability to influence the conformation of neighbouring sequences that cannot adopt the Z or Z' configuration.

We thank Michael Becker and Peter Dervan for many productive discussions of helix structure. This is contribution no. 6198 from the Norman W. Church Laboratory of Chemical Biology. This work was carried out with the support of NIH Grants GM-12121 and GM-24393, and NSF Grant PCM79-13959. H.D. was the recipient of a NIH Predoctoral Traineeship.

Received 1 April; accepted 19 June 1980.

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Helical periodicity of DNA determined by enzyme digestion

D. Rhodes & A. Klug

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

The periodicity of DNA has been determined by binding short, stiff pieces of DNA to a flat surface and using DNase I to probe the accessibility of the phosphodiester bonds. We have found that the distance between successive DNase I cutting sites is 10.6 ± 0.1 bases for the DNA immobilized on three different surfaces. We identify this value with the number of base pairs per turn of the DNA double helix in solution.

FOR many years it was assumed that the B form of DNA, observed in X-ray diffraction patterns from fibres at high humidity, represented the structure of DNA in solution¹⁻³. The B form has 10 base pairs per turn of the helix, but recent studies have suggested a value close to 10.5 (refs 4, 5). However, the disagreement persists⁶. We describe here a new approach to the problem of the helical repeat of free DNA, which gives results of high precision.

An accurate determination of the helical periodicity of DNA in solution is required to follow in detail the folding of DNA from solution into nucleosomes, the elementary building blocks of chromatin. We have postulated⁴ a change in the DNA periodicity when nucleosomes are formed, in order to reconcile the model of a nucleosome core derived from X-ray crystallographic studies, where the DNA in each nucleosome is wound into about two turns of a superhelix, with measurements of the

superhelicity on closed, circular DNA extracted from SV40 chromatin, which showed a change of linkage number nearer one⁷.

Our approach is based on that used previously to probe the helical periodicity of DNA on the nucleosome⁸⁻¹¹. The action of DNase I (and some other nucleases) on chromatin or on nucleosome cores is to produce, after denaturation of the DNA, a set of single-stranded fragments differing in size by about 10 bases. Presumably the enzyme tends to attack the regions of the DNA backbone which are well exposed, and so will produce a spectrum of fragment sizes, which will repeat with approximately the periodicity of the DNA double helix. Liu and Wang¹² found a similar distribution of DNase I fragments when studying DNA interacting with gyrase, and also observed it in the case of DNA bound to hydroxylapatite. This work, together with the development by Lutter⁹ of a high resolution gel electrophoresis

system capable of resolving random sequence DNA fragments differing in size by a single nucleotide, stimulated the experiments described in this article.

A double-stranded DNA fragment, nominally 140 base pairs long, has been immobilized on a flat, inorganic surface, then lightly digested with DNase I and the fragments produced separated by the denaturing, high resolution gel system. The distance between successive DNase I cutting 'sites', that is the distance between neighbouring groups of accessible phosphodiester bonds, is found to be 10.6 ± 0.1 bases. Because of the simple geometry of the experiment, and because the same value is found for the DNA bound to three different inorganic surfaces, we believe that the measured distance between DNase I cutting sites directly reflects the helical periodicity of DNA free in solution.

Design of the experiment

The idea behind the experiment is that if a straight, stiff piece of DNA lies on an atomically flat surface, the access of the enzyme to one side of the DNA helix is hindered in a uniform way along its length (Fig. 1). Only the most exposed phosphodiester bonds will be accessible to the enzyme, and these will recur with the periodicity of the double helix (Fig. 2b). The distance between the sites of attack of the nuclease gives the helical repeat directly. In the absence of a hindering surface all phosphodiester bonds are equally accessible and no 'image' of the periodicity is seen.

There are two methods for measuring the periodicity of the nuclease cutting sites. The first is to use a piece of DNA of defined length and incorporate a radioactive label at, say, the 5' terminus. Then the length of a single-stranded fragment produced by the nuclease action gives the position of cutting from the 5' end, and the intensity of the band on the autoradiograph gives essentially the frequency of cutting at that site. A kinetic analysis of the cutting rate can be made⁸. For this method to work, all the DNA molecules must lie in the same azimuthal orientation relative to the surface, and this assumption is justified by the results. The second method, which does not require a fixed length of DNA and is independent of the orientation of the molecule, is to introduce the label after the nuclease digestion, but this gives less total information (see below).

As a source of short, random sequence, double helical DNA pieces of defined length, we have used '140-base pair DNA' extracted from nucleosome cores, actually 148 ± 2 bases long. The persistence length of DNA is about 600 Å, so this DNA behaves like stiff rods^{13,14}.

To immobilize the DNA on the surface, that is to stop it rolling about, inorganic crystals were chosen which naturally formed flat surfaces, and where divalent cations were present or could be supplied. To exclude the possibility of a particular surface imposing a particular periodicity on the DNA by epitaxial action, experiments were performed with three different materials having different surface structures, namely calcium phosphate, magnesium phosphate and mica. To minimize interactions between the DNA molecules, fine crystalline dispersions were used so that the binding surface areas were very large in comparison with the concentration of DNA.

Materials and methods

Double helical DNA was extracted with water-saturated phenol/chloroform (1:1 v/v) from preparations of nucleosome cores⁸, precipitated twice with ethanol and the precipitate dissolved in 20 mM Tris-HCl, pH 8.0, and 20 mM NaCl. The length distribution of the single strands ranged from 146 to 150 bases with an average of 148 bases.

The 5' termini of the 140-base pair DNA were labelled with ³²P by incubating with [γ -³²P]ATP (Amersham) and T4 polynucleotide kinase (Boehringer) essentially as described by Lutter^{8,9}. The efficiency of labelling was not less than 50%. When DNA was digested with DNase I before end-labelling, the DNase I fragments were labelled with ³²P as described else-

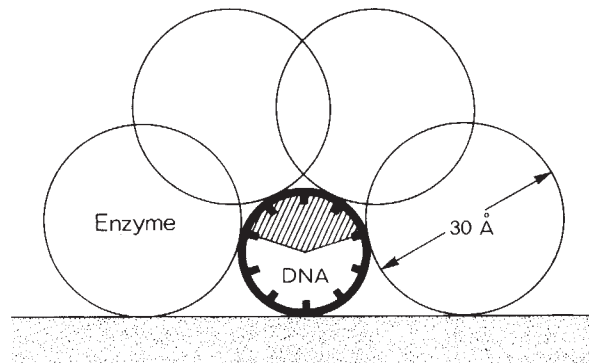


Fig. 1 Schematic drawing of the accessibility of an enzyme to DNA lying on a flat surface. The surface restricts a spherical enzyme with a diameter of 30 Å to a maximum of four accessible phosphodiester bonds. (DNase I, if spherical, would be larger than shown and have even less access to the DNA.)

where⁸. To be able to quantify the binding of ³²P-labelled DNA to a surface, the excess [γ -³²P]ATP was separated from the labelled DNA fragment on a non-denaturing, 6% polyacrylamide gel⁸.

The calcium phosphate and magnesium phosphate surfaces were prepared by mixing potassium phosphate with either CaCl₂ or MgCl₂ to a final concentration of 40 mM potassium phosphate and 25 mM CaCl₂ or 25 mM MgCl₂ in 50 mM Tris-HCl, pH 8.0. Magnesium phosphate readily forms platelet-like crystals whereas calcium phosphate forms a polycrystalline precipitate. The mica surface was prepared by grinding up mica sheets, washing and resuspending the powder at 0.2 g ml⁻¹, in 50 mM KCl, 8 mM MgCl₂ and 20 mM Tris-HCl, pH 8.0. Similar conditions were used to bind DNA to mica for electron microscopy¹⁵.

A typical experiment consisted of adding 5 µg ³²P-5'-end-labelled 140-base pair DNA to 50 µl of a suspension of calcium phosphate or magnesium phosphate or mica, and leaving for 1–2 h at room temperature. The extent of binding can be monitored by pelleting the suspension and measuring the radioactivity in the pellet.

Incubations with DNase I (Worthington) were carried out in conditions where all the DNA was bound to the surface. In the experiments using calcium or magnesium phosphate, 10 U DNase I was used at 37 °C for 5–20 s, but with mica only 0.5 U DNase I was needed to obtain the same extent of digestion. Incubations were stopped by adding EDTA in excess of the divalent cation. The DNA was extracted with chloroform/isomyl alcohol (24:1 v/v) and precipitated twice with ethanol. DNase I digests of the parent nucleosome cores, which were used as size markers, were carried out as reported elsewhere⁹.

Samples of both DNase I-digested 140-base pair DNA and nucleosomes were fractionated by 6–10% denaturing high resolution gel electrophoresis, which is capable of resolving random sequence DNA fragments that differ in size by only one base⁹.

Experiments with 140-base pair DNA

The results of a typical experiment where the 5'-end-labelled 140-base pair DNA preparation was bound to calcium phosphate, magnesium phosphate and mica, and then digested with DNase I are shown in Fig. 3, lanes a–c. In the autoradiograph a pattern of fine bands can be seen, which represent single-stranded DNA fragments differing in size by only one base. The intensities of the fine bands vary sinusoidally, showing broad maxima at regular intervals, about 10 bases apart.

The 'ladder' formed by the broad bands or maxima is an image of one side of the DNA structure as seen by the nuclease. A group of successive phosphodiester bonds, which are accessible to DNase I, give in the autoradiograph a set of intense fine bands. This set of fine bands, if unresolved, would give a broad

band, or envelope, whose centre corresponds to a local maximum in the frequency of cutting on the DNA. We call this a 'site'⁹.

The distance between DNase I cutting sites can be obtained by simply counting the number of fine peaks between the maxima, corresponding to sites, and then dividing by the number of sites traversed. In Fig. 4 the number of fine peaks between sites S3 and S9 is 63, giving a value of $63/6 = 10.5$ bases. A more objective analysis of the densitometer tracings using Fourier methods, described later, gives 10.6 ± 0.1 bases as the distance between consecutive DNase I cutting sites.

Note that the absolute lengths of the fragments, which are determined by including known length markers in the gel separation (Fig. 3, lane N), and which locate the sites with respect to the 5' end, have no role in this determination.

Interpretation of the DNase I cutting pattern

We have interpreted the above results as if most of the DNA molecules lie in the same azimuthal orientation on the surface, and also as if both DNA strands were being cut at exactly the same distance from each respective 5' terminus.

The simplicity of the DNase I pattern in Figs 3 and 4, which show maxima with halfwidths of 4–5 bases and about 10 bases apart, suggests that these assumptions are valid. After allowing for the distribution of DNA lengths present in the original preparation⁹, the width of the DNase I cutting site is only about 2 bases. This is what one might expect when the access of a molecule, with a minimum diameter of 30 Å, to the DNA helix is hindered by a flat, backing surface (Fig. 1). Moreover, as either or both of the two 5' termini in a molecule are labelled at random, what is observed is the composite frequency of cutting on the two strands of the double helix. The two strands must be cut in a symmetrical fashion to give rise to such a clean pattern. This means that the two ³²P-labelled 5' termini bear the same relationship to the binding surface, which is geometrically equivalent to saying that the unique dyad through the centre of the DNA molecule lies perpendicular to the surface (Fig. 2*b*). If this were not the case and the orientations of the molecules were

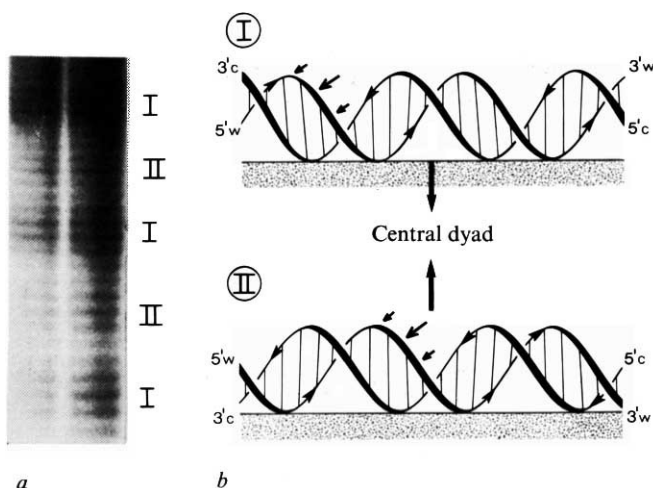


Fig. 2 Two settings of DNA on the surface. *a*, Autoradiograph from a region of a denaturing, high resolution gel electrophoresis of a sample showing two patterns of band maxima, I and II, displaced relative to each other by five bases. *b*, Schematic drawing of the two possible azimuthal orientations of a DNA molecule bound symmetrically to a surface. Setting I represents the major azimuth of the DNA, giving rise to the DNase I fragments as also observed in Fig. 3, lanes A, B and C. Setting II represents the minor azimuth of the DNA, giving rise to a set of DNase I fragments which migrate halfway between the major set of DNase I fragments. The piece of DNA in II is rotated through 180° with respect to I. A site of DNase I cutting is represented by three arrows. (This site need not occur at the most exposed position, furthest from the surface, as the enzyme need not cut at the site of binding.)

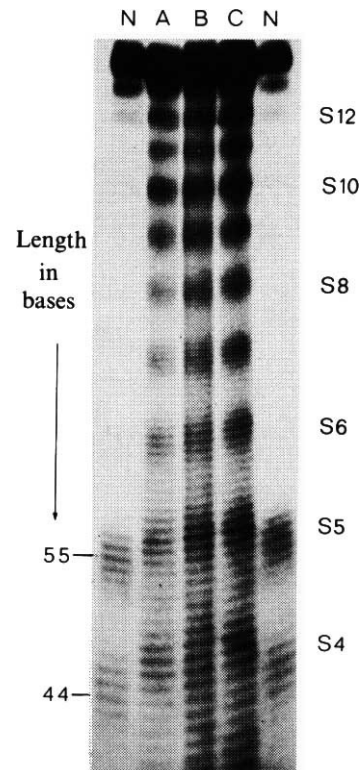


Fig. 3 Autoradiograph of DNA fragments from electrophoresis in a high resolution, denaturing gel made with 8% acrylamide and 1.35% methylene-bis-acrylamide. Lanes A, B and C contain DNA from DNase I digests of the ³²P-end-labelled 140-base pair DNA preparation bound to calcium phosphate (A), mica (B) and magnesium phosphate (C). Lane N contains DNA from DNase I digests of ³²P-end-labelled nucleosome cores. Broad bands in lanes A, B and C, corresponding to sites of DNase I attack, are labelled S4 to S12, while the absolute fragment length in bases is indicated for lane N on the left.

random, then each 5' end of the two strands in a molecule would give rise to its own series of bands, but displaced with respect to each other. The maxima in the cutting pattern would then be much broader than we have observed.

Although the results speak for themselves, it seems at first sight unlikely that all the DNA molecules would lie down in the same orientation. While clearly the bulk of the energy of binding to the surface comes from the phosphates along the length of the molecule, these would be indifferent to the orientation adopted. However, the binding of the ends of the molecule depends on the particular orientation and presumably it is the need to balance this interaction at the two ends which decides the symmetrical orientation adopted.

Further reassurance that the DNA molecules dispose themselves symmetrically on the surface comes from some experiments where a second, fainter pattern of bands can be seen lying halfway between those in the normal pattern, also repeating about every 10 bases (Fig. 2*a*). We believe the second pattern comes from DNA molecules which have rotated through 180° with respect to the first setting, so that the 5' termini are still symmetrically disposed, and the central dyad is still perpendicular to the surface. This rotation would give an apparent translation of half of the periodicity, about 5 bases (Fig. 2*b*, I, II). When the second setting is present, never at more than about 30%, then counting between maxima becomes more difficult, but the Fourier analysis still gives the same value of 10.6 bases. That it is the ends which determine the particular orientation is made clear by an experiment in which the 140-base pair DNA preparation was treated with nuclease S1, which would remove any bases projecting at the 5' end. This treatment results in both settings I and II appearing with equal proportion.

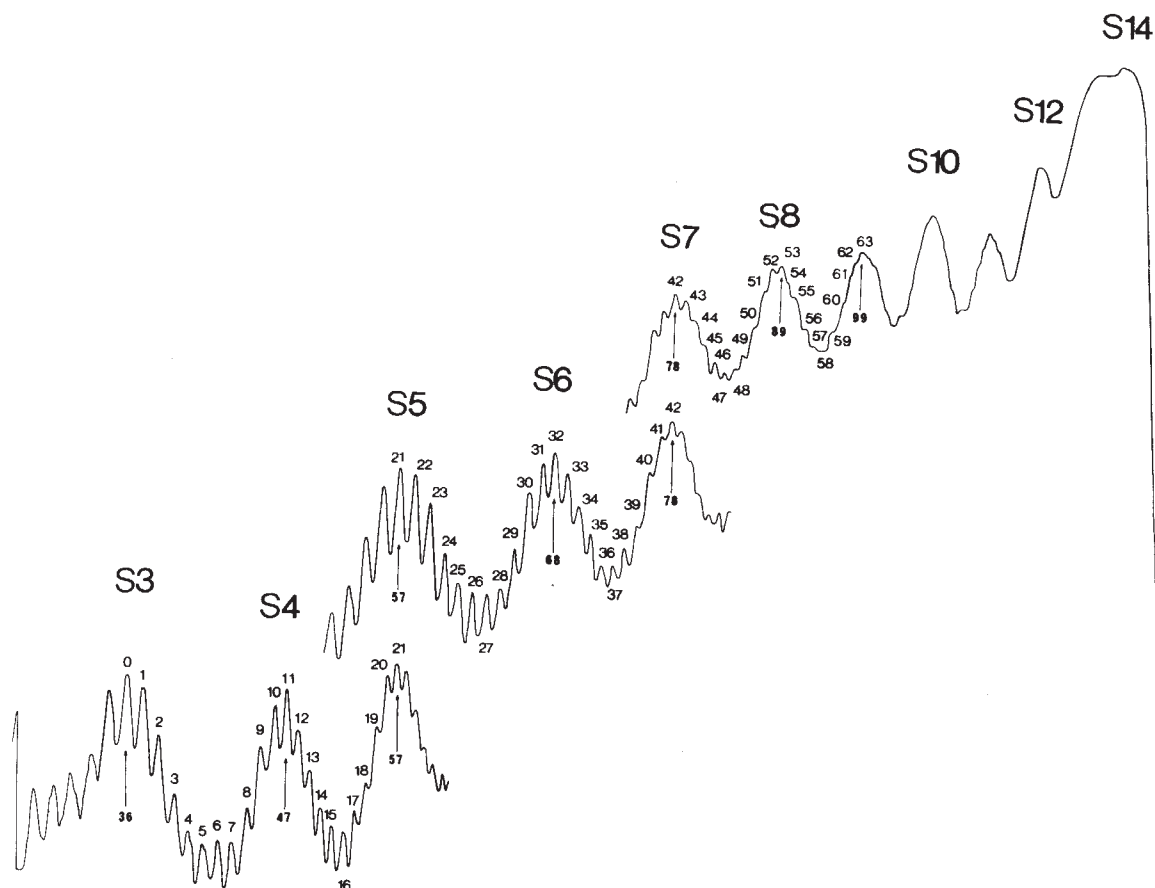


Fig. 4 Densitometer tracings of autoradiographs from the high resolution gel in Fig. 3, lane A (S5–S14), and of the same sample electrophoresed for a shorter time (S3–S5). The fine peaks, which are produced by DNA fragments differing in size by one base, have been numbered from 0 to 63 over the five bands from S3 to S9. The absolute fragment length in bases is indicated under the broad peaks in the densitometer tracing.

Probability of DNase I cutting at the sites

The probability of cutting at each site on the 140-base pair DNA can be found by cutting out the broad bands from the gels and measuring the radioactivity in each. However, the percentage of radioactivity in a band must be corrected for any cutting which might have occurred between that site and the labelled 5' end⁸.

The result is shown in Fig. 5. The probabilities of cutting at each of the 13 sites are approximately equal, but there is a clear tendency for an alternation between successive sites. This is to be expected, as in a DNA helix with a screw of 10.6 bases, there is almost an exact integral repeat after two turns. This local alternation in cutting frequency can indeed be seen directly in Figs 3 and 4, in the region of sites S9 and S13. Apart from this small modulation, analysis of Fig. 5 shows that all sites are essentially equivalent and cut at random, again justifying one of the assumptions behind the experiment.

Fourier analysis of the cutting frequency

The gel patterns may be analysed by Fourier transformation, which is more objective and accurate than simply counting the number of fine peaks between maxima, corresponding to sites. Moreover, it is the only method applicable when there are superimposed patterns with different periodicities or, what is more germane (see below), several patterns with the same periodicity but different starting points.

A Fourier transformation program applied to the amplitudes of the fine peaks in a densitometer tracing of an autoradiograph of a high resolution gel produces a frequency spectrum of the periodicity (ies) present. The results of the method applied to such densitometer tracings as given in Fig. 4 are shown in Fig. 6. The breadth of the central maximum and the side lobes are just

what would be expected from the limited length of pattern used in the analysis, in this case five repeats. The good agreement with the theoretical function expected (of the form $\sin 2\pi NX/2\pi NX$ where N is the number of bases and X the periodicity in 'reciprocal bases') indicates that a single waveform is making up the DNase I patterns of Figs 3 and 4.

The frequency spectra given by the experiments with the 140-base pair DNA bound to the three surfaces agree well with each other. The mid-points at half height are: calcium phosphate, 10.58 bases; magnesium phosphate, 10.6 bases; and mica, 10.62 bases. These values coincide well within the experimental error, which we estimate is 0.1 base. We conclude that the distance between DNase I cutting sites reflects a property of the DNA structure that is conserved when the DNA is bound to different inorganic surfaces.

Experiments with DNA of different lengths

To eliminate the possibility that the helical periodicity of 10.6 bases per turn of DNA found above could somehow depend on the particular length or end distribution of DNA used, we have also investigated a preparation of nominally 200-base pair DNA pieces extracted from nucleosomes (rather than from nucleosome cores). This contains a range of sizes, from 140 to 240 base pairs. With such a wide distribution of lengths, the external 5' end-labelling technique loses its simplicity. The radioactive label is now introduced after the nuclease treatment, to label the new 5' ends of the fragments produced ('internal labelling').

The result of such an experiment is shown in Fig. 7, together with that of a 140-base pair DNA preparation treated in the same way. A pattern of bands with intensity maxima at about

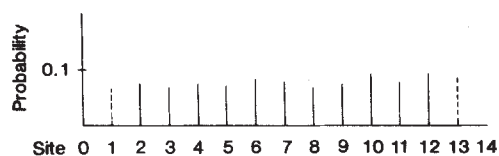


Fig. 5 The normalized probability of DNase I cutting at each site on the 140-base pair DNA fragment bound to calcium phosphate. S1 and S13 are shown dashed as these values are less accurate than the values at the other sites. The diagram is for the early stages of digestion, when the average number of cuts per single strand is 0.98.

every 10 bases is seen, but this has a higher background than the patterns shown in Fig. 3. The difference between the two experiments is that in Fig. 3 and Fig. 7, channel A, the only fragments seen are those beginning at the ^{32}P -labelled 5' end and ending at a DNase I cut, whilst in the case of the '200-base pair DNA' (Fig. 7, lane D) all the fragments produced by DNase I are seen because the ^{32}P label was introduced after incubation with the enzyme. The pattern obtained by internal labelling does not depend on the azimuthal orientation of the DNA molecules. The first experiment gives a direct map of the cutting sites, whereas the second gives the autocorrelation function containing a superposition of similar patterns, all with different starting points.

Thus in Fig. 7, lane D the gel scans do not show a clear sinusoidal pattern, so direct counting of peaks between maxima is more difficult. The Fourier method gives a frequency of cutting of 10.8 ± 0.3 bases (not shown). This value is, however, less reliable than that given by the external 5'-end-labelling method, because it is weighted by the shorter fragments which occur in greater number than the long ones, whereas in the 'external' method all fragments occur in equal proportions (in the early stages of digestion). Even the 140-base pair DNA piece, of defined length, gives a less accurate value when the internal cuts, rather than original 5' ends are labelled. However, in the gel, the ladder of band maxima from the 200-base pair DNA (Fig. 7, lane D) lines up well with that of the 140-base pair DNA (Fig. 7, lane E), showing that the periodicity of DNase I cutting is independent of the length of DNA used.

In these two patterns, each fragment is produced from two DNase I cuts. Thus, the distance between neighbouring DNase I cutting sites can be obtained simply by dividing the absolute length of a fragment (hitherto not required) by the fragment number (Fig. 7). This method gives a periodicity of DNase I cutting of 10.6 bases, in agreement with the results obtained from the external labelling experiment.

Consistency of absolute size of fragments and the periodicity

The ladder of broad bands produced from DNase I fragments labelled after digestion (Fig. 7, lanes D or E), lies nearly halfway between the ladder of DNase I fragments produced from the 5'-end-labelled 140-base pair DNA preparation (Fig. 7, lane A). This tells us that, for this particular length of DNA, the origin of the series of DNase I cutting sites is 4.5 bases away from its 5' terminus (compare 2b, I). This value, together with the values of the absolute lengths of the fragments found in the experiment shown in Figs 3 and 4, provides a check on the helical periodicity. For instance, the centre of site S5 is at 57.3 bases (Fig. 4), which would give the origin of DNase I cutting at $57.3 - 5 \times 10.6 = 4.3$ bases, from the 5' end, in very good agreement with the independently measured value.

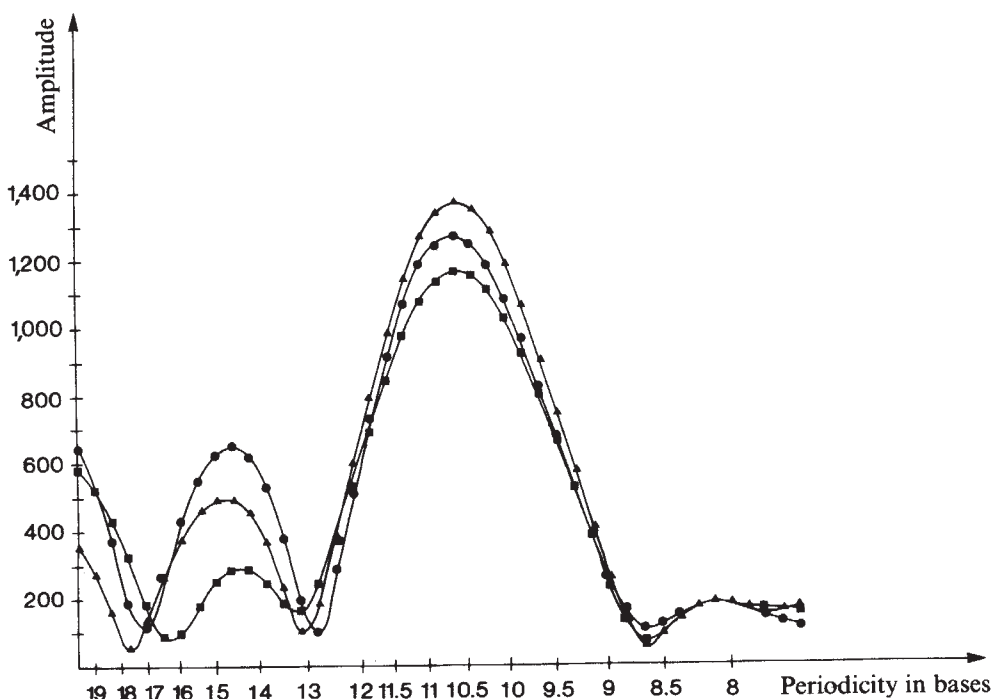
Other measurements of the helical periodicity of DNA

It has long been recognized that crystal packing tends to produce an integral number of base pairs per turn of a helix, permitting repeated regular contacts along the fibre length¹⁶.

Prompted, in part, by the issue raised by us⁴, Wang used a novel method to determine the helical repeat of DNA in solution⁵. This makes use of the effects of oligonucleotide insertions on the superhelicity of closed, circular DNA. He found a value of 10.4 bases per turn, which is close to the value of 10.6 bases we have found. Theoretical calculations by Levitt have suggested that straight DNA free in solution is most stable with 10.6 base pairs per helical turn rather than 10 (ref. 17).

The DNA fibres have been re-investigated by Zimmerman and Pfeiffer⁶ who concluded, from X-ray diffraction studies, that highly swollen fibres still had the 10 base pairs per turn of the

Fig. 6 Frequency spectrum of DNase I cutting patterns computed by Fourier transformation. ^{32}P -end-labelled 140-base pair DNA fragment bound to: $\blacktriangle$, calcium phosphate; $\blacksquare$, magnesium phosphate; and $\bullet$, mica. In each case the mid-point at half height of the main peak occurs at 10.6 bases. 107 points (53 bases) were used in the analysis for all three surfaces.



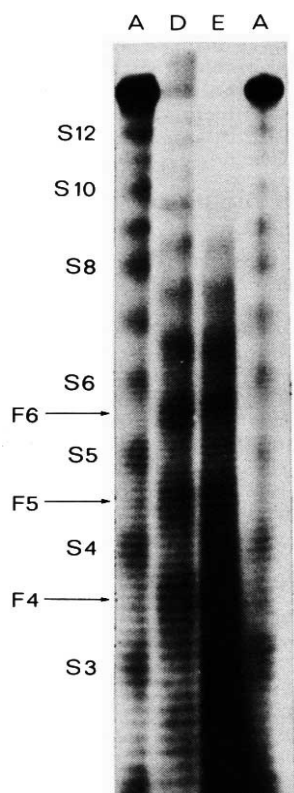


Fig. 7 Autoradiograph from a high resolution, denaturing gel made with 8% acrylamide and 1.35% methylene-bis-acrylamide. Lane A contains DNA from DNase I digests of the ^{32}P -end-labelled 140-base pair DNA preparation (external label). Lane D contains DNA fragments from DNase I digests of the 200-base pair DNA preparation, labelled after digestion (internal label). Lane E contains DNA fragments from DNase I digests of the 140-base pair DNA preparation, labelled after digestion. Broad bands in lane A corresponding to DNase I sites are labelled S3 and S12, while F4 to F6 indicate the DNase I fragments in lanes D and E. In all three experiments the DNA was bound to calcium phosphate.

helix. In our laboratory, J. T. Finch and L. C. Lutter (unpublished) have carried out X-ray studies on partially oriented gels of short stiff pieces of DNA, 150–300 base pairs long, in which the interparticle centre-to-centre distance was about 30 Å, but still found a helical repeat of 10 base pairs. Clearly these results disagree with our finding of 10.6 base pairs per turn of the DNA helix in solution. We can only conclude that in these gels or fibres of DNA, the molecules still interact, however weakly, through several layers of water and influence each other to produce an integral screw. It is relevant that structuring of water on the surface of clays appears to decay with a distance of about 10 Å (ref. 18).

Other measurements of the helical repeat are less reliable. Circular dichroism measurements and small angle X-ray scattering of DNA in solution have been used but the results are questionable because of the complexity of the data^{19,20}. Griffith²¹, from an electron microscope study, has reported a pitch of 10.5 base pairs, but in view of the systematic study by Vollenweider *et al.*²² on the effects of dehydration during sample preparation, it is unlikely that the measurements made by Griffith referred to DNA in the B form.

Finally, it is known that the helical periodicity of DNA in solution is influenced, to some extent, by cation type and concentration, and temperature^{23,24}, but the effects on our

results would amount to changes of not more than 0.1 base pair. In our experiments the cations and the ionic strength have been somewhat different, for the DNA bound to the three different inorganic surfaces, but nevertheless the same helical repeat was obtained.

Relationship to the DNA in nucleosome cores

The knowledge of the helical periodicity of DNA in solution is not only of intrinsic interest, but has important implications for the supercoiling of DNA in chromatin. To resolve the linkage number paradox, referred to in the introduction, we⁴ suggested that the helical periodicity of DNA changes from 10.4–10.7 base pairs in solution to about 10 in the nucleosome. The value of 10.6 base pairs we have found for the helical periodicity of DNA in solution agrees with this prediction. However, the prediction was dependent on taking the value of 10 base pairs per turn for DNA wrapped on the nucleosome core, the value then given by Noll²⁵ from his DNase I digestion studies.

Ironically however, more accurate measurements show that the average distance between DNase I cutting sites on the DNA in nuclei²⁶ and on the nucleosome⁹ is about 10.4 bases, rather than 10. Nevertheless, as pointed out in ref. 26, the periodicity of DNase I cutting, although closely related to the structural periodicity, need not be the same. This is because of the geometry of the nucleosome structure, where all the cutting sites on the DNA do not offer the same environment to the enzyme. In fact, it was concluded by Prunell *et al.*²⁶ that DNase I fragments from nucleosome DNA, that are multimers of 10.4 bases, could have been produced from a helical repeat of 10 base pairs. This question will be further discussed elsewhere (L. C. Lutter and A. K., in preparation).

In contrast to the case of DNA on the nucleosome, the geometry of our experiment, with the DNA lying on a flat surface, is simple. The accessibility of each turn of the double helix to the enzyme should remain constant along the length of the DNA fragment. This is confirmed by the result that the probability of cutting at successive sites is essentially the same (Fig. 5), quite unlike the results for DNA on the nucleosome⁸. We therefore conclude that the distance between DNase I cutting sites, on DNA immobilized on a surface, directly reflects the helical periodicity. Moreover, because we have found the same value of 10.6 ± 0.1 bases for three different surfaces with different atomic geometry, we identify this value with the number of base pairs per turn of the DNA double helix in solution.

We thank Drs P. J. G. Butler and R. A. Crowther for helpful discussions, and Dr. Crowther for writing the Fourier transformation program.

Received 27 February; accepted 28 May 1980.

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LETTERS

The complex radio galaxy 4C47.51 (1919+479)

J. G. Robertson

Netherlands Foundation for Radio Astronomy, PO Box 2, 7990 AA Dwingeloo, The Netherlands

An observation of the source 1919+479 with the Westerbork Synthesis Radio Telescope (WSRT) at $\lambda = 21$ cm has shown it to have a remarkable morphology, probably that of a very unusual and very large wide angle tail radio galaxy. It is situated in a cluster of galaxies at an estimated redshift of ~ 0.1 . Although distortions of the morphology of radio sources caused by a cluster environment are well known, for example in producing normal head-tail and wide angle tail sources, the observed structure of 1919+479 is shown here to differ in several respects from previously known cluster sources.

1919+479 was included in a study^{1,2} of 4C sources situated in the direction of Zwicky clusters of galaxies, in which observations at 2,695 MHz with the NRAO 300-ft telescope and the three-element interferometer (operated as a synthesis instrument) were presented. The former showed that the overall size was about 8 arc min, the latter showed a component coincident with the associated optical galaxy, and two other hot-spots. The Zwicky cluster in this case was Zw1916.8+4855 (which is of exceptionally large angular extent, nearly 20° , ref. 3). However, inspection of the optical field has shown that the galaxy identified with 1919+479 is almost certainly situated in a background cluster (see below).

The present observation was made in July 1979, at which time the WSRT consisted of 13 available telescopes along a 1.3 km east-west line. Details of the telescope and standard data reduction procedures are given elsewhere⁴. One 12 h observation was sufficient to obtain data on baselines from 36 m to 1,296 m with an increment of 36 m. This resulted in a grating ring radius of 20×27 arc min ($\alpha \times \delta$) and, with the usual tapered illumination of the aperture, a synthesized beam of 26×35 arc s. After Fourier transformation of the data, the CLEAN algorithm⁵ was applied, and the resulting total intensity map is shown in Fig. 1. The noise level in this map is 0.48 mJy per beam area. The map has been corrected for the attenuation due to the primary beam of the individual telescopes; however, this is only 7% at most for the emission from 1919+479.

The galaxy previously proposed² as the optical identification is marked in Fig. 1. The radio structure is clearly very unusual. The diffuse region labelled F exhibits an approximately constant opening angle of $\sim 47^\circ$, but is abruptly cut off at its far end in a straight line perpendicular to the ridge line. Connecting this component to the galaxy at C is a jet or tail which has two sharp bends. Between C and D the jet is unresolved, but in region E it has a half-power width of ~ 20 arc s. The western lobe of the source includes the bright component B and a 'tail' A. Although in gross morphology the lobe BA resembles an independent head-tail galaxy, and has no detectable emission connecting it with the eastern complex, it is almost certainly part of the same source. This is because inspection of the Palomar Sky Survey reveals no galaxy of $m_R \leq 18$ in the region of hot-spot B or its northern extension. Identification with a galaxy fainter than this limit would imply an unrealistically large size (≥ 4 Mpc) for the lobe. Moreover, the map of polarized intensity (prepared from the present WSRT observation) shows that the emission from region B is $\sim 12\%$ polarized, in contrast to the low degrees of polarization normally found near the parent object (for example, $\sim 1\%$ for component C). The 'head' B is also clearly extended both along the head-tail axis and perpendicular to it

(the latter at region B1), unlike normal head-tail sources. Finally, the integrated flux densities of the two lobes are similar (see below). If this interpretation is correct, 1919+479 is a very unusual wide angle tail galaxy.

Of the three components shown in the NRAO interferometer map², two are confirmed by the present observation (B and C), while the third, located to the south-west of the weak source south of the apex of component F, is not confirmed.

No measured redshift is available for the galaxy identified with 1919+479. However, if the galaxy is assumed to have an absolute magnitude of $M_p = -21.7$ ($H_0 = 75 \text{ km s}^{-1} \text{ Mpc}^{-1}$), typical of the galaxies associated with wide angle tailed radio sources in clusters⁶, then its apparent magnitude (on the PSS red plate) of ~ 14 mag implies a redshift of ~ 0.1 . Using this estimate, the projected extent of both lobes is ~ 700 kpc. This places 1919+479 among the largest known cluster sources. The integrated flux densities are $S_{1412} = 0.47 \pm 0.03$ Jy and 0.61 ± 0.03 Jy for the eastern and western lobes respectively, giving a total luminosity at 1.4 GHz of $1.6 \times 10^{24} \text{ W Hz}^{-1} \text{ sr}^{-1}$, typical of tailed or complex sources. Integration of the contour diagram from the NRAO 300-ft observation² shows that $S_{2695} = 0.57 \pm 0.03$ Jy, giving a spectral index $\alpha_{1.4}^{2.7} = 1.0 \pm 0.1$ ($S \propto \nu^{-\alpha}$). The equipartition magnetic field⁷ in component F is $\sim 1.7 \mu\text{G}$ and the minimum energy density in relativistic particles and magnetic field is $\sim 2 \times 10^{-13} \text{ erg cm}^{-3}$ (taking the emitting volume as a cone). If this component is confined by thermal pressure of a hot intracluster medium (ICM), then $nT \geq 280 \text{ cm}^{-3} \text{ K}$, where n is the number density of protons in the ICM, and T is its temperature. In the diffuse component A the equipartition field is $\sim 2.2 \mu\text{G}$ and the minimum value of nT required to confine the component laterally is $\sim 660 \text{ cm}^{-3} \text{ K}$. These values are typical of large diffuse radio source lobes (irrespective of whether or not the source is located in a cluster of galaxies)⁸⁻¹⁰.

On the Palomar Sky Survey red plate the galaxy identified with 1919+479 is seen to be surrounded by a group or cluster of galaxies: there are five galaxies within 1 arc min and about 10 within 3 arc min. They are 2-3 magnitudes fainter than the radio source identification, consistent with their being typical cluster galaxies at a redshift of ~ 0.1 , while the identified galaxy is the

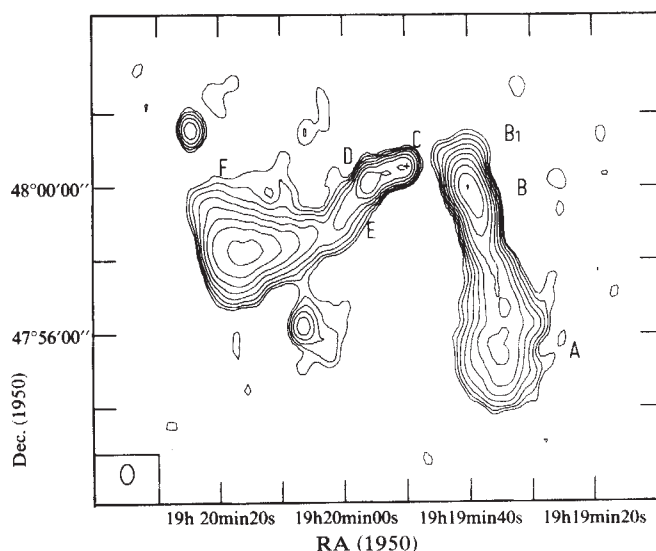


Fig. 1 Total intensity distribution of 1919+479 at 1412 MHz. The contour levels (logarithmically spaced) are 0.9, 1.5, 2.5, 4.2, 7.0, 11.5, 19.5, 32.5, 54.0 and 90 mJy per beam area. Letters identify components of the source discussed in the text; +, the position of the associated galaxy. The inset ellipse represents the half-power beam.

dominant member of the cluster. The total extent of the cluster is difficult to determine because of the low galactic latitude (15.2° , that is well below the latitude for completeness of the Abell catalogue¹¹) and because of possible confusion with fainter members of the overlying very extended Zwicky cluster. However, there are ~ 55 possible members within a radius of 15 arc min (the Abell radius for a cluster of redshift ~ 0.1). The cluster could, therefore, be as rich as Abell's¹¹ richness class 0 or perhaps 1, but further optical observations will be required to examine the cluster membership of the various galaxies. Radial velocity information from measured redshifts of the galaxies is also needed for more detailed modelling of the interaction of the radio source with the surrounding medium. The two small diameter radio sources to the north and south of component F have no optical identifications on the PSS.

In comparison with other complex extended sources, 1919+479 has a large size (unless the adopted redshift is a serious overestimate). With lobe sizes of ~ 700 kpc and an overall extent of ~ 1.2 Mpc, this source would considerably exceed the size of previously known large cluster sources such as IC711 (550 kpc, ref. 12), 1709+397 (460 kpc, ref. 13) or even the regions of diffuse emission in Abell 2256 (~ 650 kpc, ref. 8) or the Coma cluster (~ 450 kpc, ref. 14). Only the giant radiogalaxies such as 3C236 (~ 4 Mpc, ref. 15) and 3C326 (1.7 Mpc, ref. 9), which are not situated in clusters, significantly exceed the estimated size of 1919+479. The gap in the emission between the galaxy at C and the nearest part of component B/B1 is also large, although not exceptionally so. Its length is ~ 140 kpc, much larger than the gaps of 3–10 kpc commonly found near the heads of head-tail galaxies^{16,17}, and greater than that in wide angle tail sources such as 3C465 (25 kpc, ref. 18) but of the same order as the gap between the nuclear source and the western component of 1200+519 (~ 90 kpc, ref. 13) if the wide angle tail interpretation of the latter source is adopted. A further similarity between these two sources is the existence of a continuous tail or jet of emission extending from the galaxy to the second lobe. The physical processes responsible for the morphology of these two sources may therefore be similar, although it should be noted that the overall size of 1200+519 is only 220 kpc, and neither of its lobes is individually exceptional.

To return to the morphology of 1919+479, there are several interesting relations between the two lobes. First, a line passing through hot-spot D and the galaxy also passes perpendicularly through a noticeable protrusion B1 in the western lobe. (This feature is well above the noise level and is seen very distinctly in a map made with no tapering of the observations in the aperture plane.) This line is likely to be the axis of recent activity. Moreover, an extension of the ridge line in the triangular component F passes through the peak of the hot-spot B and is parallel to the line DCB1. This would be consistent with simultaneous ejection of the material which has given rise to the prominent components F and B, and with projected motion of the galaxy in an approximately northward direction being responsible for the overall wide angle tail morphology. The clearest problem which this source poses to interpretation within present models is the formation of the uniformly diverging region F, with its termination along a straight line. Projection effects may play a part in the appearance of this component, but for them to be a major influence the long axis of the component must lie almost along the line of sight, which would imply an extremely large physical size. An unusual three-dimensional structure seems unavoidable. Another interesting feature is the one-sided jet CDE. The presence of a surrounding medium (presumably an intracluster medium) would seem to be essential to explain the observed morphology, but making detailed models of this source will be possible only after observations at other frequencies become available, enabling the spectral index and polarization properties to be examined in detail, and after a reliable redshift is found from optical observations.

The Westerbork Synthesis Radio Telescope is operated by the Netherlands Foundation for Radio Astronomy with the financial support of the Netherlands Organization for the

Advancement of Pure Research (ZWO). I thank Dr R. D. Ekers, Dr R. G. Strom and Professor Dr H. van der Laan for comments, Dr J. O. Burns for pointing out that 1919+479 lies in a background cluster, and R. Windhorst for assistance in the preparation of overlays for optical examination of the field.

Received 13 February; accepted 3 June 1980.

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IUE observations of the atmospheric eclipsing binary system ζ Aurigae

Robert D. Chapman

Laboratory for Astronomy and Solar Physics, NASA-Goddard Space Flight Center, Greenbelt, Maryland 20771

ζ Aurigae is an eclipsing binary star system with a property shared by only a few other systems. It consists of two stars in orbit around one another: a cool supergiant star (spectral type K2 II) and a hot main sequence (spectral type B8 V) star. The supergiant star is nearly 200 times larger than the Sun, while the main sequence star is only about seven times larger than the Sun. The Earth lies in the orbital plane of the two stars, and thus they periodically eclipse one another. When the relatively small B star goes into eclipse behind the cool supergiant star, its light passes through extended outer gaseous layers—the outer atmosphere—of the cool star. Thus the B star is an astrophysical light source which serves as a probe of the atmosphere of the K star. The eclipses, which occur every 972 days, have been well studied at visible wavelengths^{1,2}. We report here IUE observations of the ζ Aur stellar system carried out between September 1979 and January 1980. A preliminary look at the data shows the advantage of UV observations of the system. There is strong evidence for cool, outflowing material near the primary star and hotter material farther from the primary perhaps in a shockwave. The onset of the atmospheric phase of the eclipse of the system—difficult to predict because of the irregularities of the supergiant atmosphere—began between 15 September and 1 November 1979.

As the B star goes into eclipse behind the K star, one wishes to study spectral lines produced by absorption of the B-star photospheric light by the K-star atmosphere. At roughly 4,300 Å the two stars are equally bright, and thus at this and longer wavelengths the photospheric light of the K star makes interpretation difficult. However, at UV wavelengths the K star is much fainter than the B star, and interpretation of the observations is greatly simplified. In the IUE case, with spectra in the wavelength range 1,200–3,200 Å, the K-star continuum is negligible, and we can observe spectral lines produced by the extended K-star atmosphere with no contamination from the K-star photospheric spectrum, throughout the entire wavelength range.

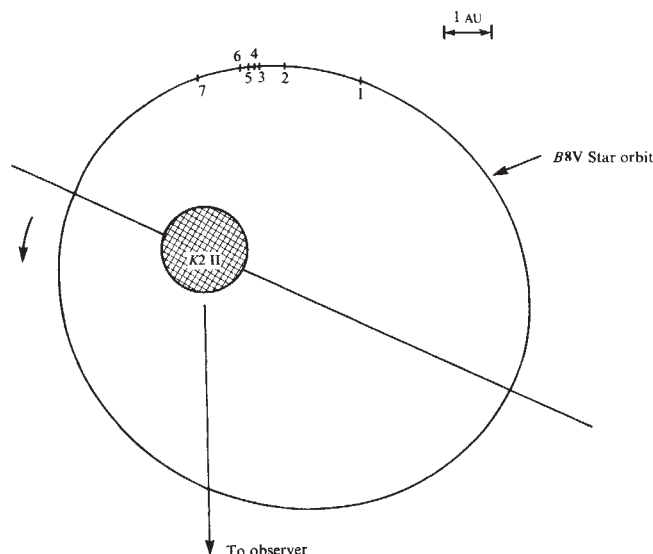


Fig. 1 Schematic diagram of the ζ Aur system.

Spectra have been obtained when the *B* star was as the positions numbered in Fig. 1. The 1979 dates of the observations are: (1) 15 September; (2) 1 November; (3) 13 November; (4) 15 November; 18 November; (6) 22 November and (7) 16 December.

The spectrum obtained on 15 September, a pure absorption spectrum, looks very similar to the spectrum of the *B7+B8* star 64 Orionis, supplied by D. S. Leckrone (see Fig. 2). The major differences between the ζ Aur spectrum and the 64 Ori spectrum is the presence of very strong multi-component absorption lines of Si II (1,260 Å, 1,264 Å), Si IV (1,394 Å, 1,403 Å), C IV (1,548 Å and 1,551 Å), and the presence of Mg II resonance doublet (2,795 Å and 2,802 Å) with strong P Cygni profiles.

The profile of the Mg II doublet Fig. 3 shows the classical P Cygni shape, indicative of an expanding shell. The absorption component of each line appears to show a reversed profile, characteristic of the strongest solar lines such as the Ca II, H and K lines. However, the appearance is undoubtedly due to two absorption lines being present, probably because of a double shell. This conclusion is supported by the spectra taken during

the total phase of the eclipse. If the peak in the absorption line were chromospheric emission from the *K* star—as one finds in the case of the reversed Ca II-line profiles—then that central emission should remain visible during total eclipse even after the continuum and the absorption lines have vanished. In fact, the central emission is not present and, therefore, it must have been continuum showing between two partially resolved absorption lines.

The spectra obtained between 1 and 18 November show a large number of absorption lines not present in the 15 September spectrum. The absorption lines increase in number and strength during the time period (Fig. 4). The 1 November spectrum has been studied in some detail, and a large fraction of the absorption lines have been identified. Ionic spectra which have changed from 15 September to 1 November, showing that they arise from an extended atmosphere or dense wind region surrounding the *K* star include Al II, Al III, Cr II, Mn II, Fe II, Ni II and Ca II. This wavelength region includes the resonance lines of many of these ions. The majority of the lines correspond to lines observed in the UV solar emission line spectrum from Skylab^{3,4}. The C IV, Si IV and Mg II lines observed in the 15 September spectrum are also visible in the 1 November spectrum but are very little changed in appearance. We conclude that the high ionization potential lines are formed far from the *K* star, whereas the group of low-ionization-potential lines which show significant changes are formed in cool plasma nearer to the *K* star: probably a cool wind. These cool wind lines seem to be shifted longward relative to the centre of mass of the binary system. Thus, the outflow must be dynamically influenced by the *B* star.

The high ionizations potential lines are typified by the C IV lines (Fig. 5). Each of the C IV lines consists of a narrow line, near the rest wavelength, and a longward shifted broad line. If one looks at the entire sequence of C IV lines, the narrow lines clearly remain unchanged, whereas the broad lines increase in strength into the eclipse. I believe that the narrow, constant line is probably interstellar, while the broad line is formed near the *B* star, perhaps in a shockwave produced by the *B* star moving supersonically through the *K*-star wind. Highly turbulent motion in the shockwave can account for the breadth of the absorption line.

On 22 November the *B*-star continuum had disappeared, and an emission line spectrum was present. The emission lines, which include C II, Si II, Si III, Si IV, S II, Mn II, Fe II, Ni II, correspond closely to the absorption lines observed in earlier

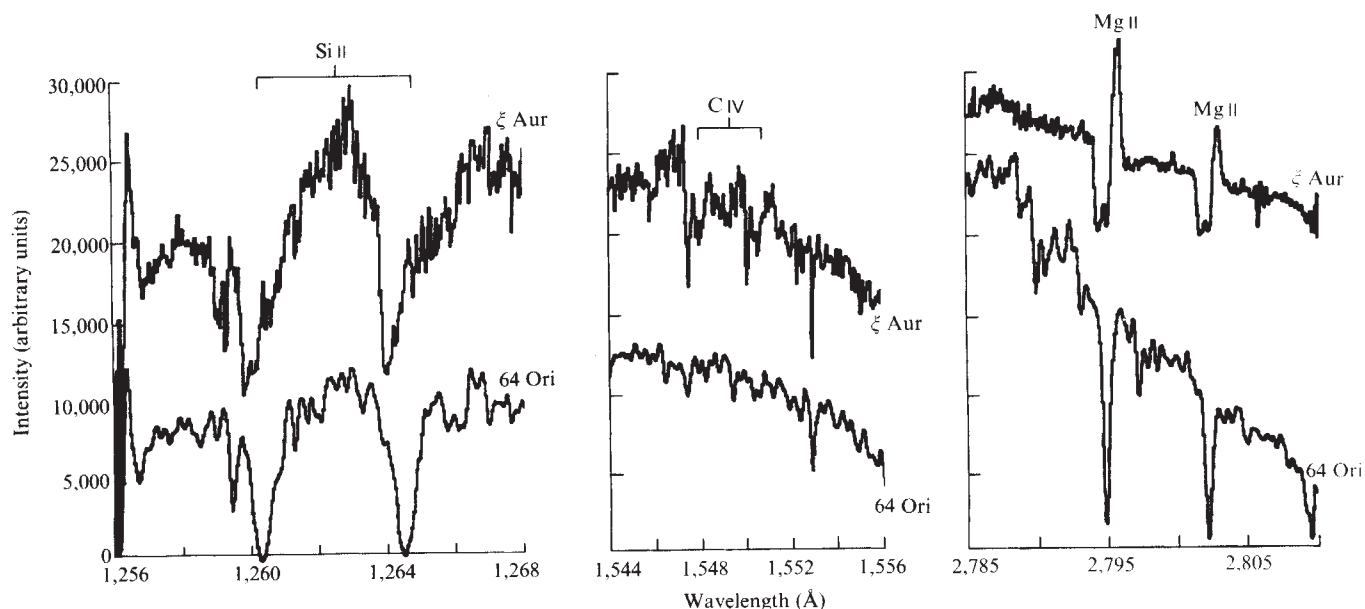


Fig. 2 Comparison between spectra of ζ Aur and the single *B8 v* star 64 Ori.

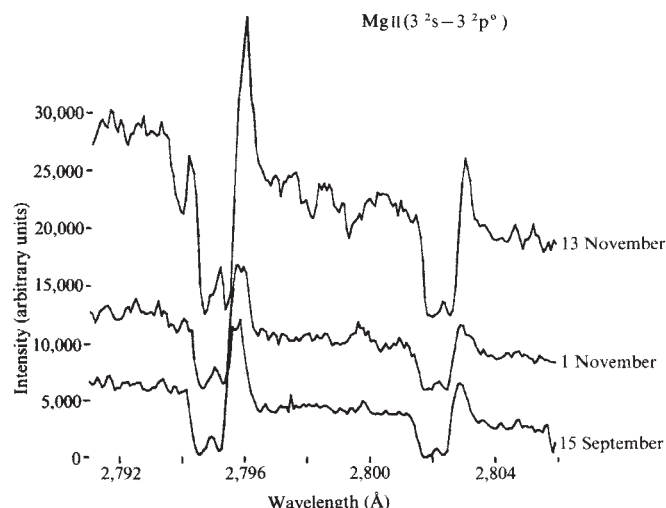


Fig. 3 The Mg II resonance doublet lines show strong P Cygni profiles. The appearances of the lines change very little with time, as can be seen from these three samples. The amplitude increase on 13 November is an exposure effect. On 22 November, the continuum level was too faint to record, and only the emission remained.

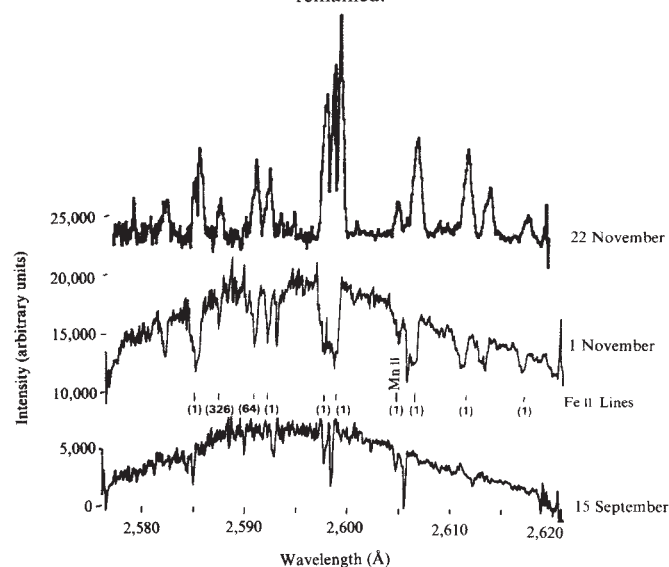


Fig. 4 Three exposures showing the region of the Fe II resonance lines. Between 1 and 22 November 1979, the absorption lines continue to increase in width. Multiplet number is indicated on figure.

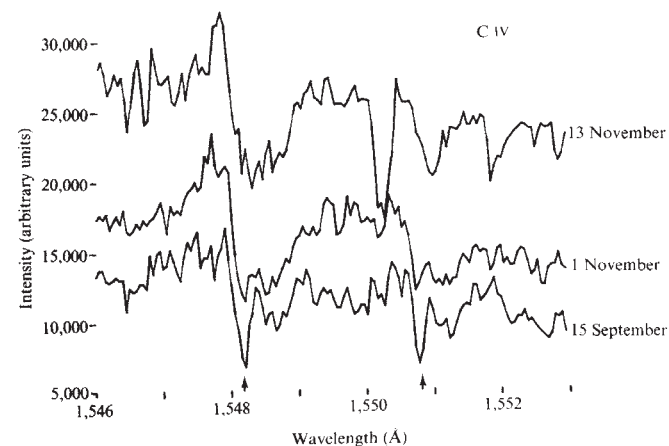


Fig. 5 The spectrum region of the C IV resonance lines. Each line seems to consist of a shortward feature and a longward feature. The longward feature is broader and shallower than the shortward. By 13 November, the features have blended together. Arrow marks laboratory wavelength.

spectra. We have searched for intercombination lines that have been observed in many stellar shell spectra, particularly in the low ionization stages of oxygen. None were found. The density of the material is therefore quite high, probably in excess of 10^{12} cm^{-3} .

We are now embarking on a more thorough study of the ζ Aur data, including additional observations during total eclipse and egress. Full details will be published elsewhere.

We thank the project scientist and observatory administrator for the opportunity to carry out this project, and the resident astronomers and staff of the IUE observatory at the NASA-Goddard Space Flight Center for practical assistance.

Received 15 January; accepted 18 April 1980.

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IR observation of a persistent meteor train

M. A. Hapgood

Department of Physics, University of Southampton,
Southampton SO9 5NH, UK

A long-lived (>32 min) persistent meteor train has been observed with a low light television camera which was sensitive only to radiation between wavelengths of 700 and 900 nm. The peak emission from the train was about 2×10^{17} photons $\text{s}^{-1} \text{ m}^{-1}$ and the meteor which produced the train had a magnitude corrected to the zenith of about -6 . It is suggested here that this IR emission arises from the excitation of the atmospheric band of molecular oxygen ($b^1\Sigma_g \rightarrow X^3\Sigma_g$) by the sodium catalytic process, a process which Baggeley¹⁻³ has previously proposed to be responsible for the visible radiation from persistent meteor trains.

The persistent train was recorded between 01.30 and 02.02 UT on 19 September 1979 from the Jungfraujoch Observatory in the Swiss Alps ($46^\circ 33' \text{N}$, $7^\circ 59' \text{E}$, 3,570 m). The train was observed with an image isocoon television camera used in integration mode to image structure in the near IR airglow. A picture was produced every half second and was recorded on a time lapse video tape recorder. A camera tube with a photocathode having an extended red (S-25) photocathode spectral response was used and a Schott RG715 filter was placed over the front of the camera lens to eliminate visible light. Thus the effective spectral region of the camera was confined to a region between 700 and 900 nm as shown in Fig. 1 (obtained by combining manufacturers' specifications).

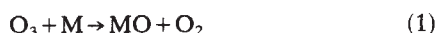
The meteor which produced the train appeared at 01.30.14 (± 1 s) UT. The luminous trail started outside the field of view, entered at the upper edge (elevation 17°) and ended in the middle of the field (elevation 12°). The range of the meteor from the camera may be estimated at 300 ± 50 km. As the meteor travelled 2.9° in 0.5 s at the estimated range of 300 km the velocity $v \geq 30 \text{ km s}^{-1}$. Comparing the brightness of the meteor with Jupiter recorded under similar conditions gave m_v of -3 for the brightest part, which was at an elevation of 14° . Using the correction given by Lovell⁴, the magnitude of the meteor adjusted to the zenith was about -6 .

The train which persisted after the passage of the meteor originated from a part of the meteor trail at elevations between 14.0 and 14.5° . Taking the range as 300 km it follows that the train lay between 15 and 20 km above the end height of the meteor. Assuming that the end height lay between 70 and 80 km suggests that the train lay at heights between 85 and 100 km.

During the period of observation the persistent train moved eastwards across the field of view. The upper and middle parts of the train moved very rapidly. By 02.02 UT they were 18° east of the original azimuth which indicates a drift velocity of $50 \pm 10 \text{ m s}^{-1}$. The lower part of the train became drawn out behind the main body of the train and parallel to the drift direction. The drift velocity of the trailing edge of the train was estimated to be about 20 m s^{-1} . Thus there was considerable wind shear at the lower end of the train. This shear and the comparatively high velocity above it suggest that the train lay well above the mesopause and confirm the range of heights deduced from elevation measurements.

The brightness of the train was estimated by comparison with field stars of spectral class A. At the beginning, the brightest part of the train was comparable to a star of magnitude +5 but by the end of the period of observation it was barely visible above the limiting magnitude +7. To convert stellar magnitudes into photon emission rates we assume that the stars radiate as black bodies. Thus in our spectral passband a fifth magnitude star of class A₀ gives a photon flux at the camera of $2 \times 10^8 \text{ m}^{-2} \text{ s}^{-1}$. As we used a fairly narrow spectral passband, we neglect the differences between the spectra of the stars and the train and take the photon flux from the train to be $2 \times 10^8 \text{ m}^{-2} \text{ s}^{-1}$ at the camera. This flux came from a portion of the persistent train which corresponded to a single resolution element in the picture (about 0.2° of angular extent, equivalent to 1 km at the estimated range of 300 km). By assuming that the meteor train was at right angles to the line of sight we obtain a line emission rate from the persistent train of $2 \times 10^{17} \text{ photons s}^{-1} \text{ m}^{-1}$. This estimate will be seriously in error only if the meteor were travelling in a direction close to the line of sight. This seems unlikely as the meteor train was of significant angular extent ($>6^\circ$).

The depth of the Earth's shadow⁵ in the region of the train at the time of observation was about 1,000 km so the light was generated in the train rather than being scattered sunlight. The light from such self luminous trains is thought to be generated by a cyclic chemical process first suggested by Chapman^{6,7}:

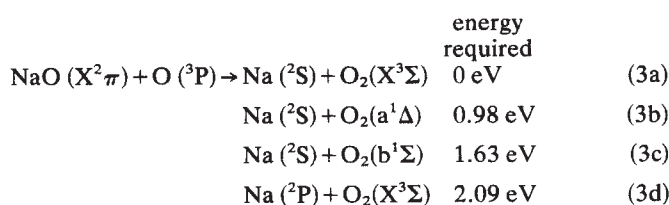


Metal atoms (M) released by the meteoroid catalyse the conversion of atomic oxygen and ozone to diatomic oxygen. If the reactions are sufficiently exothermic the products may be in excited states and emit photons as they decay to their ground states. The process may continue almost indefinitely as the metal

atoms are recycled and the energy is extracted from the atmosphere. Baggaley has studied the case of sodium¹⁻³ for which both reactions have high rates⁸: a significant proportion of the sodium atoms produced by reaction (2) are in the excited ^2P state which decays by D line emission. Baggaley³ estimated the production rate for the ^2P state from data on the sodium nightglow and found that to produce a clearly visible persistent train (one emitting $>5 \times 10^{15} \text{ photons s}^{-1} \text{ m}^{-1}$) a meteor of $m_v > -2$ is required. Because almost all persistent trains ($>96\%$) are produced by meteors brighter than $m_v \sim -2$ Baggaley concludes that the visible light emission from persistent trains can be explained solely by this process—the sodium catalytic process.

The present radiation detected from the persistent train cannot be sodium light. We therefore consider whether the oxygen molecules in reactions (1) and (2) may be produced in excited states. The $\text{b}^1\Sigma_g$ state of molecular oxygen, in the absence of quenching, decays to the ground state emitting radiation in a band between 700 and 900 nm, as shown in Fig. 1. (The (1,0) and (0,0) bands are not shown as these connect with the vibrational ground state of O_2 and so are lost to a ground-based observer by absorption in the atmosphere.) The transition is forbidden for electric dipole radiation and so it has a long radiative lifetime of 12 s (ref. 9). The quenching of O_2 ($\text{b}^1\Sigma_g$) proceeds mainly by collision with N_2 and has a rate constant⁹ $K = 2 \times 10^{-21} \text{ m}^3 \text{ s}^{-1}$. This leads to significant quenching¹⁰ only at heights below 85–90 km. It follows that the molecular oxygen emissions will not be quenched at the heights of the train reported here.

The production of O_2 ($\text{b}^1\Sigma_g$) is permitted in reactions involving many elements but because of the high reaction rates, the most favourable case is sodium. If we study reaction (2) for sodium, we find that, with an energy¹ for the reaction of $2.5 \pm 0.2 \text{ eV}$, there are four variations of the basic reaction which are energetically and spin allowed:



For reactions (3b) and (3c) the energy required is that needed to excite the lowest vibrational level ($v=0$) in the upper electronic state. The total energy available from the reaction is much more than this so the higher vibrational levels may be excited (up to $v=5$ for the $\text{b}^1\Sigma_g$ state). Reaction (3d) produces the sodium D line emission discussed by Baggaley and reaction (3c) produces molecular oxygen emission in the near IR. If the reaction were to take branch (3c) as often as (3d) we may expect a photon emission rate from molecular oxygen comparable to the photon emission rate from sodium. This gives an emission rate³ of $5 \times 10^{15} \text{ photons s}^{-1} \text{ m}^{-1}$ for a $m_v \sim -2$ meteor which extrapolates to $2 \times 10^{17} \text{ photons s}^{-1} \text{ m}^{-1}$ for a meteor of $m_v \sim -6$. This is in good agreement with the emission rate from the train reported here. Thus the near IR emission from the persistent train may be explained in terms of the sodium catalytic process.

I thank M. J. Taylor for help with the observations and Miss P. Rothwell for valuable discussions. The use of the Hochalpine Forschungsstation Jungfraujoch during September 1979 is gratefully acknowledged.

Received 31 March; accepted 13 June 1980.

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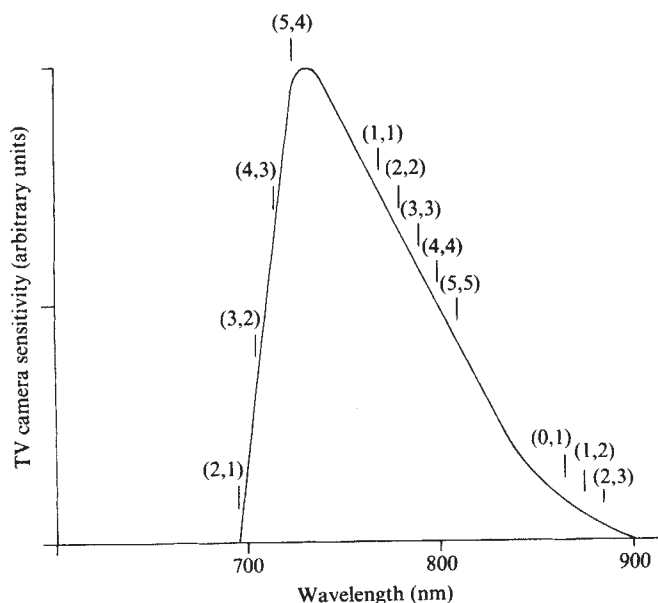


Fig. 1 The effective spectral response of the television camera and the positions of the vibrational bands of the atmospheric system of O_2 .

Photoelectrochemical conversion using reaction-centre electrodes

A. Frederick Janzen

Photochemical Research Associates, Inc., London, Ontario, Canada N6E 2V2

Michael Seibert*

Solar Energy Research Institute, Golden, Colorado 80401

Recent reports have demonstrated the possibility of using photoactive, biological membrane components in photoelectrochemical cells¹. Such systems have produced small photovoltages and photocurrents. The present studies in our laboratories have led to the attachment of a much simpler biological complex, the bacterial photosynthetic reaction centre isolated from *Rhodospseudomonas sphaeroides*, directly onto an SnO₂ electrode. The light-induced, primary-charge-separation processes which occur across the reaction-centre macromolecule have been coupled to the electrode, and in a two-electrode configuration photovoltages of 70 mV and photocurrents of 0.3 $\mu\text{A cm}^{-2}$ have been observed in an external circuit. The phenomena are not due to Dember effects, and 'reactor-centre electrodes' may serve as a model system for future photovoltaic devices.

Photoelectrochemical cells have used chloroplast membranes², chloroplasts^{3,4}, photosystem-I particles⁵, purple-membrane fragments containing bacteriorhodopsin^{6,7}, and bacterial reaction centres⁸. In most cases, the biological component was incorporated into liposomes or bilayer lipid membranes, and in some cases the stability was enhanced by using polymers or lipid-impregnated filters. Because the interiors of liposomes are inaccessible to contemporary electrodes, definitive electrochemical studies are very difficult. Lipid bilayers often trap solvents and have small working surfaces. Furthermore, the geometry necessitated by lipid bilayer systems (two half-cells separated by the bilayer) would be quite cumbersome in a solar energy conversion device. In contrast, chloroplast membranes^{2,3} have been deposited directly onto the surface of an electrode, thus forming a simpler photoelectrochemical cell.

Bacterial reaction-centre complexes are the simplest biological units that carry out the primary photochemistry of photosynthesis. They have been isolated from photosynthetic bacterial membranes and are relatively stable at high concentration in room temperature and light conditions. A reaction centre⁹ is an 80,000–100,000 molecular weight complex containing three protein subunits and six chlorophyllous chromophores, and it carries out a multistep charge separation process with a quantum efficiency of one. In the case of *Rhodospseudomonas sphaeroides* reaction centres, 15.2% of the incident solar energy (air mass 1.2) is available as the chemical potential of charge separation at the level at which the bacteriochlorophyll dimer donor (BChl₂) is oxidized and the iron/quinone acceptor (X) is reduced¹⁰. This estimate assumes that the sample is of sufficient optical density to absorb all incident light between 380 and 865 nm. Several investigators^{11,12} have suggested that reaction centres might be deposited onto electrodes as model systems for future solar energy conversion devices. The first successful direct electrical coupling of an isolated photosynthetic reaction centre to an electrode is described here.

Using the method of Dutton *et al.*¹³ we isolated reaction-centre complexes from the purple, non-sulphur photosynthetic bacterium *Rhodospseudomonas sphaeroides* R-26 (a mutant

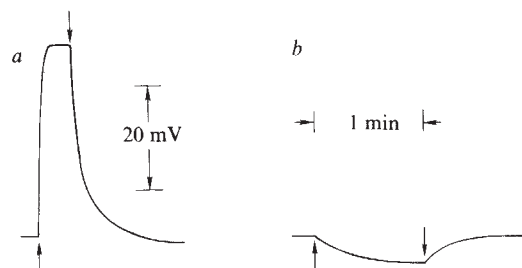


Fig. 1 The open-circuit photovoltage produced by a reaction-centre-coated, SnO₂ electrode exposed to red light ($\sim 40 \text{ mW cm}^{-2}$) in an electrochemical cell. *a*, Freshly-coated electrode. The electrode acts as a photocathode since the midpoint potential of the X acceptor in the reaction centre is more oxidizing than the flat-band potential of SnO₂. The kinetics of the photovoltage can vary with the sample. *b*, Autoclaved electrode. Upward and downward arrows indicate light on and light off, respectively. The photovoltage produced by an uncoated, SnO₂ electrode in these conditions was $< 1 \text{ mV}$.

strain of ATH 2.4.1 lacking highly unsaturated carotenoids). The complexes were suspended in 10 mM Tris-HCl buffer (pH = 8.0) at a concentration of 266 μM . Reaction centres were transferred to working electrode surfaces by dipping the electrodes into the concentrated, reaction-centre suspension. Excess liquid was removed from the lower part of the electrodes and the electrodes were dried in the dark. Platinum electrodes were platinized using standard methods, and SnO₂-coated glass was from Corning Glass Works. The experimental cell consisted of a working electrode and either a platinized platinum or SnO₂ counter electrode. The electrolyte was 0.1 M Na₂SO₄ and 0.05 M hydroquinone in water or Tricine buffer (pH = 7.1). The light source was a tungsten lamp (Unitron Instruments), and the light passed through both a Corning CS2-62 red filter, which cuts off light below 600 nm, and a 7-cm water filter. Photovoltages and photocurrents were measured in the external circuit with a Keithley Instrument Model 177 multimeter, using various load resistors connected in parallel or series with the meter. Absorbance spectra were obtained with a Cary 219 spectrophotometer. Action spectra were measured at very low light intensity using a tungsten light source focused through a 0.25-m Bausch and Lomb monochromator (6-nm bandwidth). The reciprocal of the quantum flux necessary to elicit a 10- μV response across a 10-k Ω resistor was plotted as a function of the wavelength.

Platinized platinum was the first working electrode material we investigated. Table 1a presents the photovoltages and photocurrents obtained when reaction-centre complexes were dried as a thin film on this surface. Small open-circuit photovoltages and short-circuit photocurrents were observed routinely, and the photoeffects lasted for at least several days. If these phenomena were due to the light-induced charge separation associated with the primary processes of photosynthesis, electrodes coated with inactive reaction centres would not be expected to produce photo-induced voltages and currents. Autoclaved reaction-centre-coated platinum electrodes (Table 1b) exhibit photoeffects of the same order as unautoclaved

Table 1 Photoeffects in reaction-centre-coated, platinized platinum electrodes

Working electrode condition	Open-circuit photovoltage (mV)	Short-circuit photocurrent (μA)
<i>a</i> . Freshly coated electrode	2.1	1.07
<i>b</i> . Autoclaved electrode	3.65	0.32

The total irradiated area of the electrode was about 0.25 cm² in each case.

* To whom correspondence should be addressed.

electrodes, indicating that biologically active electron transfer is not involved. Dember effects¹ could account for the phenomena shown in Table 1.

The absence of any coupling of the primary charge separation to the electrode could be due to rapid back reactions from the electrode to the oxidized reaction centres or to quenching of excitation by energy transfer from excited reaction centres to the metal¹⁴. If this is the case, the use of semiconductor electrode materials might overcome the problem. Consequently, we made reaction-centre electrodes using SnO₂-coated glass. Figure 1a shows that a reaction-centre-coated SnO₂ electrode can typically generate an open-circuit photovoltage of 37 mV. The maximum value observed thus far is 70 mV. A maximum photovoltage of about 495 mV is possible (the difference between the midpoint redox potential of the BChl₂ primary donor, +450 mV, and that of the X acceptor, -45 mV, because BChl₂ and X are located on opposite sides of the reaction-centre molecule). Mismatching of the energy levels of the reaction-centre molecules with the energy levels of the SnO₂, charge recombination within the reaction-centre film, and a lack of reaction-centre orientation could account for the small photovoltages. Typical short-circuit photocurrents observed are ~0.3 $\mu\text{A cm}^{-2}$. The residual photovoltage after autoclaving indicated in Fig. 1b is probably due to a Dember effect.

If electrons pumped across the reaction centre during the primary photoact of photosynthesis do equilibrate with the electrode, the action spectrum of the photocurrent measured in the external circuit should correspond to the absorbance spectrum of the reaction centres. Figure 2 compares the solution absorbance spectrum, the absorbance spectrum of a thin reaction-centre film, and the action spectrum of the photocurrent. The absorbance spectrum of the film is distorted with respect to the solution spectrum due to the effects of light scattering. The action spectrum, however, follows the solution spectrum closely. The peaks in the spectra at around 800 and 860 nm are due to bacteriochlorophyll absorption, while the peak at 760 nm is due to bacteriopheophytin absorption. The quantum yield (absorbed photons) for the production of electrons by 800-nm photons was 0.002.

We conclude that charge separation generated across the reaction-centre molecule as a result of the primary photo-

chemistry of photosynthesis can be electrically coupled directly to semiconductor electrode materials (although inefficiently yet). Such reaction-centre electrodes can be used in a photoelectrochemical cell to generate photovoltages and photocurrents in an external circuit. Similar results have been obtained using electrodes on which chlorophyll or chlorophyll analogues have been deposited¹⁵⁻¹⁸. These electrodes may lead to new methods of probing the primary photochemistry of photosynthesis and may also serve as model systems for future inorganic or organic photoelectrochemical devices.

We thank J. R. Bolton and J. C. Grosskreutz for their encouragement and A. J. Nozik for his suggested improvements of this manuscript. This work was supported in part by the US Department of Energy, contract EG-77-C-01-4042.

Received 21 February; accepted 28 May 1980.

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Cross-polarization ¹³C NMR spectroscopy of whole soils

P. F. Barron*, M. A. Wilson†, J. F. Stephens*, B. A. Cornell‡ & K. R. Tate§

* CSIRO Fuel Geoscience Unit, North Ryde, New South Wales, 2113 Australia

† CSIRO Division of Process Technology, North Ryde, New South Wales, 2113 Australia

‡ CSIRO Division of Food Research, North Ryde, New South Wales, 2113 Australia

§ DSIR Soil Bureau, Lower Hutt, New Zealand

The ideal tool for the analysis of soil organic matter would enable structural group analysis to be performed on whole soil samples in a non-destructive manner. However, up till now, there have been no adequate techniques available to characterize the structure of amorphous humic compounds in the solid state. Moreover, it has not been possible to dissolve all the organic material from the soil and obtain soluble material for analysis without chemical alteration during solvent extraction or pyrolysis. Although conventional NMR spectra of amorphous solids reveal no structural information due to large dipolar interactions, the use of high power cross-polarization (CP) techniques¹ has overcome difficulties in obtaining ¹³C NMR spectra of solids to the extent that spectra can be obtained in much shorter times and organic functional groups partly resolved. We now report the first recorded ¹³C NMR spectra of whole soils and show that this technique has the potential for structural analysis of organic matter in solid soil samples.

Although the ¹³C cross-polarization technique has been well documented^{1,2}, a brief description is necessary here. Its major advantages over conventional ¹³C NMR are a reduction of the

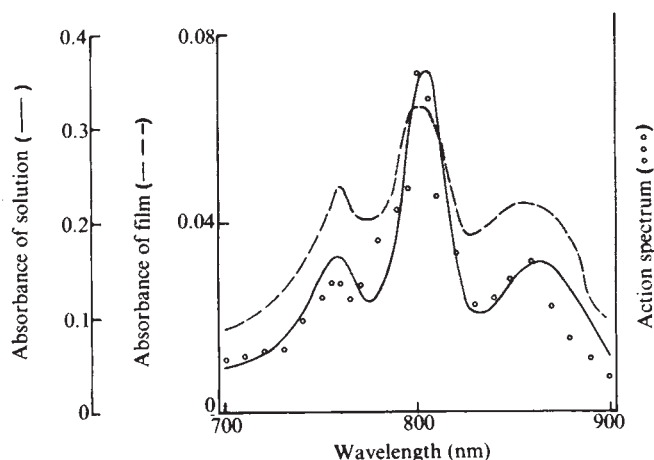


Fig. 2 A comparison of reaction-centre solution, film (six reaction centre film layers were used), and action spectra. Due to drift problems with freshly-coated, SnO₂ electrodes over the time period of the experiment, we have shown the action spectrum of an electrode aged for 3 days in the dark. In these conditions, the electrode acted as a photoanode, but the magnitude of the open-circuit photovoltage was the same as in Fig. 1a for a freshly-coated electrode. Consequently, this effect should not be confused with the small photoanodic effects of autoclaved electrodes (Fig. 1b). The action spectra of freshly-coated electrodes show the same peaks as aged electrodes, but the relative peak heights are distorted. The action spectra of autoclaved electrodes have no peaks in the 700-900 nm range.

time required to obtain a usable spectrum which, in turn, is better resolved owing to the elimination of heteronuclear dipolar broadening. The increased sensitivity to ^{13}C is achieved in two stages: the first by the transfer of polarization from the more polarized and more abundant ^1H spins to the ^{13}C spins. The maximum possible enhancement due to this effect is given by the ratio of the two gyromagnetic ratios, $\gamma_{\text{H}}/\gamma_{^{13}\text{C}}$, which is about 4. The second, and more significant, contribution results from the ^{13}C spin-lattice relaxation now occurring at the spin-lattice relaxation rate of the proton population which, in general, is an order of magnitude faster than the conventional ^{13}C rate. Thus the frequency with which data may be accumulated is greatly increased. In the presence of the high power heteronuclear decoupling field, the remaining line widths of the order of 0.2–2 kHz are due predominantly to residual ^{13}C chemical shift anisotropy (CSA)² and are not removed by the CP technique. However, the reduction in line widths is sufficient to allow partial resolution of many of the ^{13}C resonances from different functional groups with amorphous solids. The ^{13}C technique is used in the present study to determine the relative abundance of different functional groups in the organic matter in a range of soils.

To obtain this information, a number of experimental conditions must be satisfied. For example, it must be established that all carbon types approach the same degree of polarization enhancement during the CP process. This requirement is satisfied if the proton population is assumed to relax with a uniform spin temperature according to a single time constant, $T_{1\rho}$, as a result of spin-diffusion. Although the efficacy of spin-diffusion is quite short range on the time scale of the relaxation rates encountered in the soils studied here, this assumption has been widely used in the interpretation of spectra obtained from closely related coal samples which possess similar proton relaxation rates^{3,4}. A second consideration is the r_{CH}^{-6} (where r_{CH} is the internuclear distance between carbon and hydrogen) dependence of the cross-polarization rate. Clearly the non-protonated carbons will cross-polarize at a slower rate than protonated carbons^{3,4}, setting a lower limit to the contact time used to achieve adequate cross-polarization. Finally, the delay between repeating the pulse sequence must be sufficiently long to allow for the spin-lattice relaxation of the proton population.

Unfortunately, as the polarization enhancement to which the ^{13}C spin system is approaching decreases with length of contact at the rate of the ^1H $T_{1\rho}$, over-allowance in the choice of contact time will lead to reduced enhancement and increased accumulation time. Also, over-allowance in the choice of pulse repetition time will lead to increased accumulation time. Because soils have low carbon contents (<25%), both the contact and pulse repetition times must be optimised for maximum time efficiency as well as equal response of different carbon types to the CP process.

To estimate the optimum conditions for this study, Maungatua silt loam, details of which are shown in Table 1, was examined using a range of contact and recycle times. Recycle

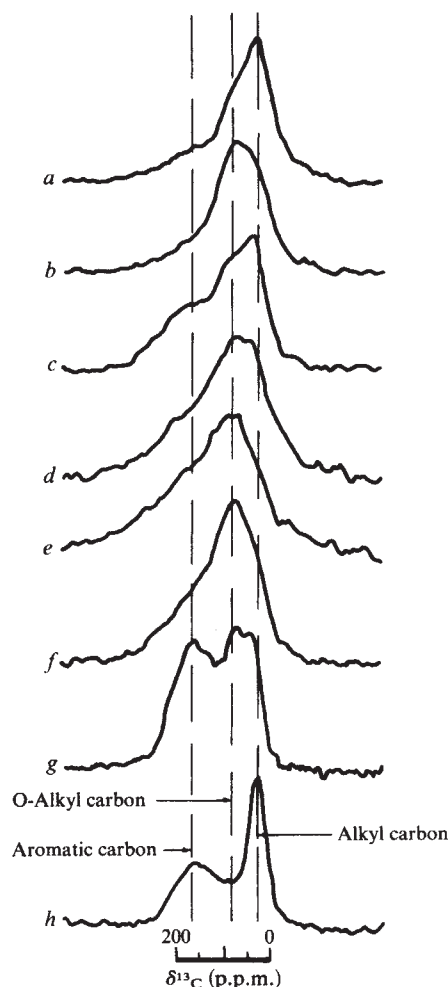


Fig. 1 CP-NMR spectra of soils and coals obtained with a Bruker CXP-100 spectrometer operating at 22.63 MHz for ^{13}C and a Bruker z-22v ^{13}C - ^1H double resonance probe. ^1H irradiation fields of 28 and 7 G for ^{13}C and ^1H respectively were used to satisfy the Hartmann-Hahn condition² necessary for cross-polarization. The number of free induction decays collected ranged from 2×10^4 to 2×10^5 . a, Maungatua humic silt loam. b, McKerrow fine sandy loam A₁ (clay-size fraction <0.02 μm , 23% organic matter) isolated by ultrasonic dispersion of soil in water and subsequent separation into sand-, silt- and clay-size material. c, Te Kopuru sand B_h horizon. d, Te Kopuru sand A horizon. e, Taupo C horizon clay concentrate (<0.2 μm , 6.3% organic matter). Conditions of isolation have been described elsewhere⁶. f, Taupo B horizon clay concentrate (<0.2 μm , 13.2% organic matter). Conditions of isolation have been described elsewhere⁶. g, Yallourn coal (woody brown coal). h, Wandoan coal (sub-bituminous black coal).

Table 1 Properties of Maungatua humic silt loam

New Zealand classification ⁵	Podzolized upland yellow-brown earth
Horizon	A ₁
%C	22.1
Cation exchange capacity me%	39.0
Total exchangeable bases me%	1.1
Parent material	Thick schist over comminuted schist
Vegetation dominants	Canopy:
	<i>Chionochloa rigida</i> 35%
	<i>Dracophyllum longifolium</i> 35%
	<i>Celmisia coriacea</i> 15%
	Ground layer:
	<i>Astelia linearis</i>
	<i>Celmisia gracilentia</i>
	<i>Cladonia aggregata</i>

times of 0.5–2 s resulted in no apparent differences in the total signal or those parts of the signal pertaining to specific carbon types, indicating that spin-lattice relaxation is very efficient for all carbon types ($T_1 < 0.1$ s). The ^{13}C - ^1H contact time was varied from 0.25 to 2 ms with maximum enhancement occurring in the range 0.5–1 ms. No change in relative enhancement of different carbon types was evident for contact times greater than 0.5 ms. Hence, cross-polarization rates are fast and the ^1H $T_{1\rho}$ is short (<10 ms), as has been found in coals^{3,4}. Thus the spectrum of Maungatua soil could be semiquantitatively interpreted using a contact time of about 1 ms and a recycle time as short as 0.5 s. All other soils were examined using a single contact of 1 ms duration with a recycle time of 1 s except for Taupo C horizon clay where a recycle time of 0.5 s was used due to the low carbon content.

Figure 1 shows typical spectra of soils and soil fractions, together with spectra of brown and black sub-bituminous coals

for comparison. Details of the other soils used in these experiments have been reported previously⁵⁻⁷. ¹³C CP spectra of coals normally consist of a relatively sharp resonance due to alkyl carbons and a broader, unsymmetrical peak to lower field, due to aromatic carbons^{3,4}. The spectrum of Wandoan coal (Fig. 1h) is typical of this. The Yallourn xylitic brown coal spectrum (Fig. 1g) exhibits an additional resonance indicative of carbons α - to oxygen in ethers and alcohols (O-alkyl), and is also obviously more aromatic. In contrast, in Maungatua soil (Fig. 1a), the dominant carbon type is alkyl with a significant amount of O-alkyl carbon also present. The aromatic carbon content seems much lower than in either coal. Figure 1c, d show the spectra obtained from the B_h and A horizons of the Te Kopuru sand. The B_h horizon of Te Kopuru sand is clearly more aromatic than the Maungatua soil and is also possibly slightly more aromatic than the A horizon of Te Kopuru sand. Similarly, more of the aliphatic carbon in the A horizon is bound to oxygen than in the B_h horizon. Thus, the chemical structure of the soil organic matter may vary with profile depth as well as soil type.

Humic and fulvic extracts of soils usually contain carboxylic acid carbon⁸⁻¹⁴ but no distinct signals were obtained downfield of the aromatic carbon signal. It is possible that the signal is hidden by the large chemical shift anisotropies of aromatic and carboxylic carbons¹. The use of the 'magic angle spinning' technique (MAS)², which removes the CSA broadening, in conjunction with the CP process should clarify this point. The brown coal spectrum exhibits a shoulder on the low field side of the aromatic signal which is possibly due to carboxylic carbon.

In the soils with low carbon content, we were able to obtain spectra by physically separating the carbon-rich clay fractions. As found for the Maungatua soil, the clay fraction of the A horizon of McKerrow fine sandy loam (Fig. 1b) is highly aliphatic, but it is also apparent that more of the aliphatic carbon is bound to oxygen. We have also obtained spectra from the <0.2 μ m clay fractions of the B and C horizons of Taupo sandy soil (a vitric andosol developed on pumice) (Fig. 1e). The C horizon fraction contained only 6% carbon and required 2×10^5 scans at 0.5 s pulse repetition to obtain the spectrum. At the field strength used (21.1 kG) the technique is probably limited to soils or soil fractions of about this carbon content or greater.

Early workers believed that humic substances were highly aromatic polycyclic materials¹³. However, recent workers have proposed different structures. Schnitzer has suggested that humic materials are highly substituted monocyclic materials in which some of the substituents are polymethylene chains of fatty acids¹⁴. Other workers⁹⁻¹² have suggested that humic materials are more aliphatic than previously thought. Grant¹⁵ has shown that polymethylene can comprise as much as 30% of the organic matter in a particular soil, while Anderson and Russell¹⁶ have offered evidence which suggests that polymaleic acid-like polymers may be important constituents of humic substances. In the soils we have investigated, our results indicate the variation in the nature of the organic matter present, from highly aliphatic to moderately aromatic.

We are continuing our studies of soil organic matter by the ¹³C CP technique with the aim of correlating the variation in structure with soil forming factors, in particular, vegetation. The combination of the 'magic angle spinning' technique with the CP process will provide more detailed and quantitative information.

We thank R. Tyler and N. Russell for soil samples and Drs A. Campbell and K. Goh for some of the soil samples. Support to P.F.B. was provided under the National Energy Research, Development and Demonstration program administered by the Commonwealth Department of National Development.

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Harmattan dust deposition in northern Nigeria

Grant McTainsh

School of Earth Sciences, Macquarie University, North Ryde, New South Wales 2113, Australia

Studies of atmospheric dust deposits originating from Africa, within Atlantic Ocean sediments^{1,2}, have provided useful indirect information on Quaternary environments, but dust studies in Africa are rare. Here Harmattan dust deposition measurements at Kano, northern Nigeria during the 1978-79 season are reported. A relation between dust deposition, visibility, solar radiation and wind speed is defined, which deteriorates during the latter half of the Harmattan season. A probable explanation of this deterioration is the local remobilization of existing dust.

The dust is thought to originate from the Faya Largeau area in the Chad Basin³ (Fig. 1). From there the dust plume travels WSW at an altitude⁴ of 600-900 m reducing visibility, relative humidity, solar radiation and temperatures along its path. The Harmattan season extends from October to April though it may vary considerably from year to year.

The dust collection technique was designed to measure the amount of Harmattan dust falling onto the ground from suspension in the atmosphere. A dust trap was set up 9 m above ground level on the roof of a building on the campus of Bayero University, Kano, isolated from local soil dust movements. The trap was a basin of distilled water 20 cm deep exposing a water surface of 0.27 m² for collecting dust. Water level was maintained daily 10 cm below the top. To prevent bird contamination of the dust trap a second basin was kept full for birds to use. The dust laden water was evaporated in laboratory ovens weekly and the residue weighed. Collections were made during three Harmattan seasons, 1976-79.

Casual observations during the Harmattan season suggest a close association between visibility and dust deposition but the

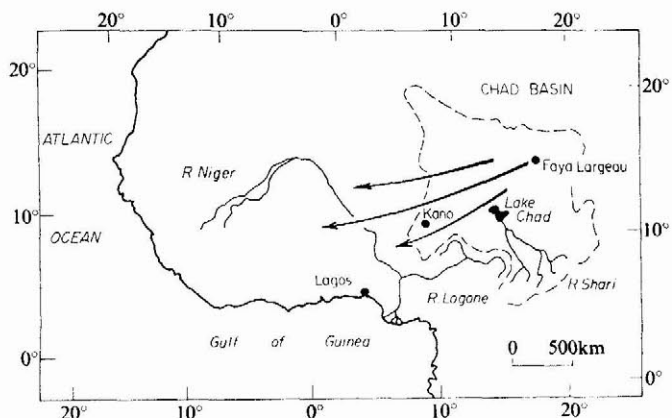


Fig. 1 The Harmattan wind in West Africa.

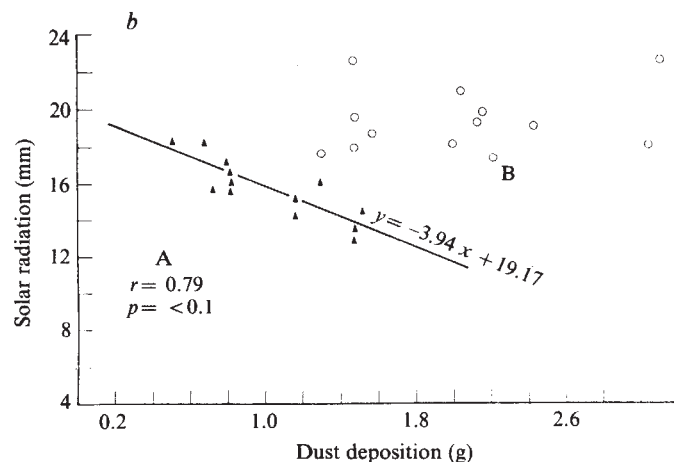
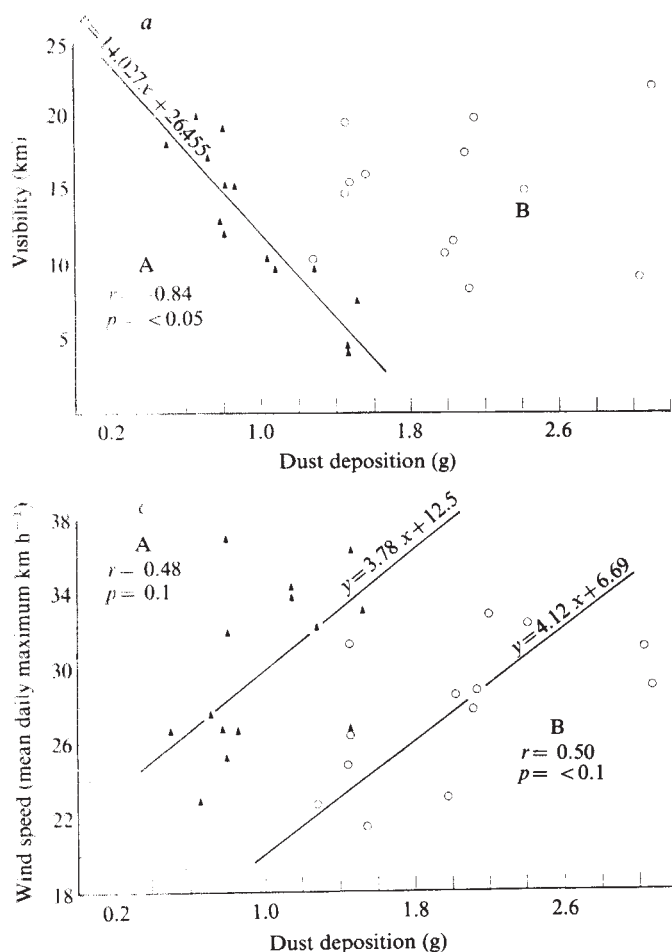


Fig. 2 a, The relation of visibility to Harmattan dust deposition—weekly mass of dust settling over 0.27 m of distilled water (see text); b, the relation of solar radiation to Harmattan dust deposition; c, the relation of wind speed to Harmattan dust deposition. Δ , Time period A (October–January); $\circ$, time period B (January–April). r is the correlation coefficient and p is the significance level found in a test of the hypothesis $r = 0$ against $r \neq 0$.

process might be accentuated by grass burning during the latter part of the dry season adding coarser particles to the measured deposition. These particles may have relatively little influence on visibility.

Therefore, measured dust deposition rates during B (after mid January) overestimate the Harmattan dust influx. The visibility–deposition regression line (Fig. 2a) is taken to indicate primary Harmattan dust deposition. Visibility data for each week during B are used to predict the expected deposition for each week. The difference between expected and measured deposition I assume to be secondary deposition.

When the corrected data are derived, total Harmattan dust deposition at Kano during 1978–79 is 99.1 g m^{-2} . This figure represents a reduction in total measured deposition of 41.7% which is the amount of secondary deposition. Based on measured dust bulk density of 0.85 g cm^{-3} the 1978–79 Harmattan dust influx represents a layer of dust 0.116 mm thick.

Harmattan dust deposition measurements made by Bromfield⁵ in the same area during 1969–72 amount to 22.6 g m^{-2} which is far lower than rates given here, and surprising during a time of drought. Bromfield collected dust in a dry deposition gauge (B.S. 1747 Part 1 1961) which can seriously underestimate deposition⁶.

Published⁷ dust accumulation rates measured on land vary from 6.6 to 20,100 mm kyr^{-1} which could in part reflect different collection and calibration techniques. A standardized method for monitoring dust is urgently needed.

With respect to historic fluctuations in dust deposition, the visibility–dust deposition relation defined here could be used to estimate dust deposition over the period for which meteorological data are available, thus providing a sensitive index of environmental change in historic times within the Sahel.

I thank Innocent Ukenna for help with the dust collections and note the recent and tragic death of Dr M. P. S. Collins of the Bayero University Harmattan Research Group. Thanks go to Bayero University for financial aid and Martin Williams for discussion.

Received 24 March; accepted 3 June 1980.

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monitored rates indicate a more complex relationship. During the first part of the season periods of low visibility are associated with high dust deposition. As the season progresses measured deposition was far greater than observed visibility would suggest. Data on visibility, solar radiation (Gunn Bellani) and wind speed obtained from Kano International Airport were averaged over each of the 28 weekly dust measurement periods of the 1978–79 season. Relationships with deposition are shown in Fig. 2.

The data consistently fell into two time periods, here termed A (12 October–12 January) and B (13 January–27 April). During A a close relation emerges between visibility and deposition and between solar radiation and deposition ($r = -0.84$ and -0.79 respectively, Fig. 2a, b). During B these relations deteriorate to statistical insignificance. The relation of wind speed to deposition remains at a lower but stable level throughout the season ($r = 0.48$ and 0.50 , Fig. 2c).

A probable explanation of these differences is local remobilization and secondary deposition of the dust during B, increasing the measured deposition and upsetting the expected relationships with visibility and solar radiation. As the remobilized dust is not derived from high altitudes the expected relations with visibility and solar radiation are disrupted, whereas during A primary deposition is dominant.

Wind speed data support this view. During A, increases in Harmattan wind speed are accompanied by greater dust deposition. During B the amount of dust deposited is higher than wind speed might suggest (Fig 2c) as wind is remobilizing and redepositing dust.

The start of the Harmattan season coincides with the end of the wet season so that during A tall grasses abound and relative humidity levels are high thus minimizing dust remobilization. As the grasses die off, relative humidity decreases and the total amount of deposited dust increases, conditions become conducive to remobilization and secondary deposition. This

Coupled effects of atmospheric N₂O and O₃ on the Earth's climate

Wei-Chyung Wang & Nien Dak Sze

Atmospheric and Environmental Research, Inc., 872 Massachusetts Avenue, Cambridge, Massachusetts 02139

Increased application of nitrogen fertilizer¹ could perturb the atmospheric nitrogen cycle and might lead to a possible increase²⁻⁶ in atmospheric N₂O. N₂O plays an important role in stratospheric chemistry⁷⁻⁹ as well as in the global radiation budget¹⁰⁻¹². Recent studies¹³⁻¹⁵ suggest that perturbation of local ozone could also significantly affect the global climate. Here we show that a doubling in the present day N₂O level might significantly perturb the distribution of O₃ and HNO₃, and that the associated climatic feedbacks from O₃ and HNO₃ perturbations could contribute as much as 0.23 K warming of the surface temperature, in addition to 0.44 K directly caused by N₂O doubling.

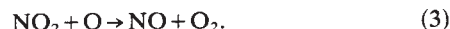
Nitric oxide (NO) is formed in the stratosphere mainly by the reaction of O(¹D) with N₂O (refs 7-9),



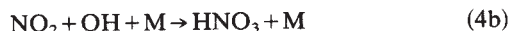
and may serve as a catalyst for O₃ removal^{16,17} mainly by



and



In the lower stratosphere ($z < 30$ km), NO and NO₂ may, however, couple with stratospheric odd hydrogen (HO_x) and odd chlorine (ClX) mainly through reactions



and



Note that while addition of NO may enhance O₃ removal through reactions (2) and (3), it may reduce the efficiency of both the HO_x cycle ($\text{OH} + \text{O}_3 \rightarrow \text{HO}_2 + \text{O}_2$; $\text{HO}_2 + \text{O}_3 \rightarrow \text{OH} + 2\text{O}_2$) and the ClX cycle ($\text{Cl} + \text{O}_3 \rightarrow \text{ClO} + \text{O}_2$; $\text{ClO} + \text{O} \rightarrow \text{Cl} + \text{O}_2$) as sinks for O₃, particularly in the region below 30 km. Thus, an increase in stratospheric NO (or N₂O) may lead to O₃ depletion

Table 1 Calculated surface temperature change ΔT_s (K) caused by a doubling of the present day atmospheric N₂O abundance, 320 p.p.b.v.

Case	Model	ΔT_s (K)
A	N ₂ O	0.44
B	N ₂ O + O ₃	0.62
C	N ₂ O + O ₃ + HNO ₃	0.67

Case A considers the sole effect due to N₂O increase. Case B includes the additional effect of O₃ perturbation (Fig. 2). Case C represents the combined effect associated with N₂O increase, including the feedbacks from perturbation to O₃, HNO₃ and NO₂. The effect of NO₂ increase is mainly to increase the absorption of solar radiation in the atmosphere²⁴, with possible greenhouse effect as it has IR bands³⁰. However, the calculated surface cooling due to the associated NO₂ increase is small, ~0.01 K. For the constant cloud temperature parameterization²⁶ with ice-albedo feedback²⁷, the calculated surface temperature change should be multiplied by a factor of 2.

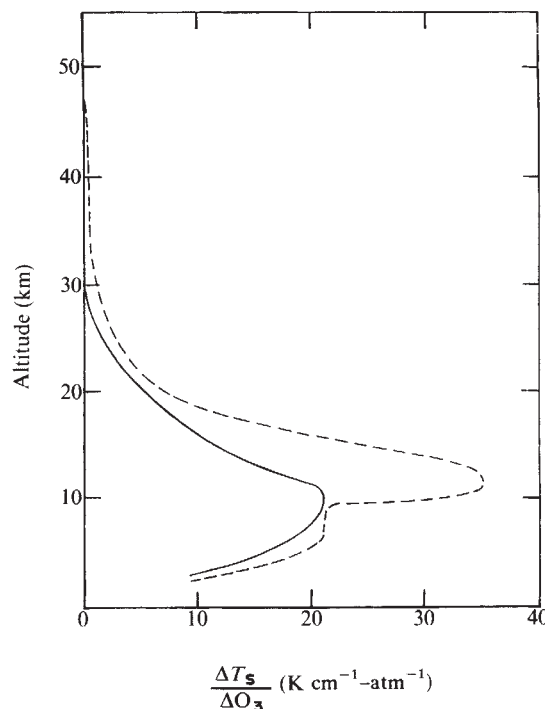


Fig. 1 Sensitivity of surface temperature change ΔT_s (K) to changes in local O₃ amount for mid-latitude (solid line) and tropical atmospheres (dashed line) (after ref. 15).

in the upper stratosphere and to O₃ increase in the lower stratosphere and upper troposphere. (For reviews of current understanding of stratospheric chemistry see refs 18-22).

Besides its dominant role in the stratospheric chemistry, N₂O has strong IR absorption bands at 4.5, 7.78 and 17 μm , and thus contributes to the 'greenhouse effect'. Calculations¹⁰⁻¹² indicate that an increase in the atmospheric N₂O abundance due to the increased application of nitrogen fertilizer²⁻⁶ might directly influence the troposphere climate.

We consider here some of the indirect climatic effects that might be associated with the compositional changes caused by N₂O increase. For instance, because of the strong photochemical coupling between N₂O, O₃ and other trace species, changes in atmospheric N₂O may perturb the O₃ distribution which also has an important role in maintaining the stratospheric thermal structure and the Earth-troposphere radiative energy balance^{13-15,23}. Figure 1 shows the calculated sensitivity¹⁵ of surface temperature T_s to changes of local O₃. Note that the maximum sensitivity occurs at around 12 km.

Furthermore, because N₂O is the precursor (reaction (1)) to stratospheric NO_x (NO, NO₂, HNO₃, NO₃, N₂O₅, ClONO₂), changes in N₂O would cause similar changes in HNO₃ and NO₂. Note that HNO₃ has IR absorption bands¹⁰ at 5.9, 7.5, 11.3 and 21.8 μm while NO₂, primarily absorbs solar radiation in the UV and visible²⁴. Consequently, changes in HNO₃ and NO₂ may also affect the global radiation budget. The combined climatic effects to be expected from a large increase of N₂O are therefore due, in part, to direct greenhouse effect of N₂O as discussed by Wang *et al.*¹⁰ and, in part, to the perturbation of atmospheric chemical composition (for example, O₃, HNO₃ and NO₂) caused by the interaction of N₂O with other trace species.

Figure 2 shows the calculated changes in the vertical thermal structure and the O₃ profile resulted from a doubling of the present day atmospheric N₂O abundance, 320 p.p.b.v. (parts per 10⁹ by volume). Note that while the calculated perturbation to column O₃ amount is relatively small (~2% increase), the changes in local O₃ amount are relatively large, up to ~+18% at 12 km and ~-19% at 38 km. The increase in O₃ below ~26 km is

mainly attributed to the strong coupling between NO_x , HO_x and ClX while the decrease above 28 km to reactions (2) and (3). The calculated changes in O_3 profile (Fig. 2) are sensitive to the choice of diffusion coefficients and the stratospheric OH concentrations which are uncertain by at least a factor of 2 or 3. In view of such large uncertainties, the quantitative aspect of the results presented in Fig. 2 should be taken with caution. The calculated changes of the column abundance of HNO_3 and NO_2 resulted from a N_2O doubling are +85% and +75% respectively. These results, on the other hand, are relatively model independent.

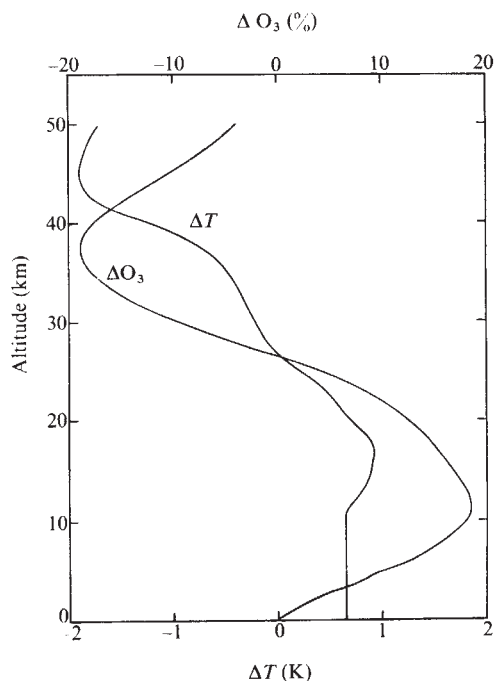


Fig. 2 The calculated changes in thermal structure and O_3 profile due to a doubling of present day N_2O abundance of 320 p.p.b.v. The calculations were based on iterative runs between the one-dimensional radiative-convective model¹⁰ and the one-dimensional photochemical-diffusive model²⁵. In the one-dimensional radiative-convective model, we used: (1) 6.5 K km^{-1} as the critical lapse rate²³; (2) constant relative humidity parameterization²³; (3) constant cloud altitude parameterization²⁶; and (4) fixed surface albedo²⁷ (that is, no ice-albedo feedback). In the one-dimensional photochemical-diffusive model, transport was treated by using the expedient concept of eddy diffusion with the diffusion coefficients taken from ref. 28. The rate data for the oxygen-hydrogen-nitrogen-chlorine chemistry were taken from ref. 22. The stratospheric H_2O mixing ratio $f_{\text{H}_2\text{O}}$ was assumed to be constant with altitude and with $f_{\text{H}_2\text{O}}$ set equal to 4.5 p.p.m.v. (ref. 22). The mixing ratio of ClX at 50 km was 2.2 p.p.b.v., which corresponds to the contributions of various known chlorocarbons to the present stratospheric chlorine level.

The combined climatic effects of the changes in N_2O , O_3 , HNO_3 and NO_2 are to decrease the temperature above ~26 km and to increase below. The stratospheric cooling is mainly associated with the local O_3 depletion because the solar radiative heating is reduced. Note that the decrease in solar heating is partially compensated by a warming effect associated with the absorption of upwelling thermal emission from a higher tropospheric temperature of about 0.67 K. The warming effect, however, seems less effective at higher altitude, as reflected by the feature that the calculated maximum cooling occurs at considerable higher altitudes than the region where O_3 depletion peaks. On the other hand, the stratospheric warming with temperature increase up to 1 K can be attributed to the

increases in both the local O_3 and the solar radiation reaching the region as a result of O_3 reduction in the upper stratosphere.

Table 1 shows the computed surface temperature change ΔT_s associated with N_2O doubling. In contrast to the partial surface cooling effect caused by the CFMs-induced O_3 perturbation^{15,29}, the effect of the N_2O - O_3 coupling provides a significant positive feedback and increases the surface temperature from 0.44 K (case A) to 0.62 K (case B). The 30% enhancement in ΔT_s is mainly caused by the calculated increases of O_3 in the lower stratosphere and upper troposphere, a region where the response of T_s to O_3 changes is maximum, as shown in Fig. 1. The associated increase in HNO_3 produces an additional positive feedback effect and increases the surface temperature further, to 0.67 K (case C). The combined climatic effect seems quite significant when compared with the one-dimensional model predicted²⁷ 2 K global surface warming due to a doubling of the present day atmospheric CO_2 concentration.

Note however, that the foregoing calculations are based on one-dimensional models in which uncertainties are large. The sources of uncertainties may arise from: (1) the inadequacies³¹ in the basic formulation of one-dimensional models; (2) the treatment of humidity, clouds and the use of critical lapse rate to approximate the vertical transport of heat by atmospheric motions²²; (3) the complexity of the stratospheric chemical reactions where those involving peroxy(HO_2) are particularly not well understood^{20,21}; and (4) the lack of present knowledge of the atmospheric N_2O cycle. Thus, the present uncertainties make it difficult to quantify the errors associated with the calculated climatic effect due to N_2O increase. Presumably some of the uncertainties discussed here may be narrowed when more atmospheric data and sophisticated models become available. Nevertheless, it seems clear that should we study the climatic effect due to a change of atmospheric 'greenhouse' material (such as N_2O) which can also influence the atmospheric chemical composition, feedback from compositional changes should be considered.

W.C.W. thanks J. E. Hansen and A. A. Lacis who initiated and participated in the development of the one-dimensional radiative-convective model. We also thank F. M. Luther for the NO_2 absorption data. This work was supported by grant NAS1-15943 from NASA Langley Research Center and by the Chemical Manufacturers Association.

Received 17 March; accepted 2 June 1980.

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Plio-Pleistocene reversal of displacement on the North Anatolian fault zone

P. L. Hancock & A. A. Barka

Department of Geology, University of Bristol, Queen's Building, University Walk, Bristol BS8 1TR, UK

Fault plane solutions¹ and the offset of geomorphic and man-made features²⁻⁴ show that present-day displacements on the North Anatolian fault zone are right-lateral (Fig. 1). The present analysis of some of the mesoscopic faults and joints cutting Upper Neogene–Lower Pleistocene continental sediments in intermontane basins between Cerkes and Erbaa indicates that there was left-lateral motion on the fault zone during part of its neotectonic evolution. The structures include conjugate reverse faults striking north-west, conjugate normal faults and joints striking north-east, and conjugate vertical joints enclosing an acute angle about a north-east trending bisector (Figs 2 and 3).

The ~1,200 km arc of the North Anatolian fault zone is generally interpreted as an early Neogene intracontinental transform boundary separating the Black Sea and Anatolian plates^{2,5} (Fig. 1a). As part of a study of neotectonic structures associated with the fault zone, we have surveyed fractures of mesoscopic scale cutting Neogene–Quaternary continental sediments in five intermontane basins between Cerkes and Erbaa (Fig. 1b). The basins are situated astride or adjacent to fault breaks which developed during six, westwards migrating, large magnitude earthquakes. The aim of investigating the mesofractures was to infer directions of compression and extension related to shear along the fault zone. Other studies of mesofractures have demonstrated their value in the kinematic analysis of major fault zones^{6,7}.

Although Neogene–Quaternary successions are different in each basin, those older than the late Pleistocene are regarded as belonging to the Pontus Formation⁸ or its lateral equivalents. The ~500 m Lower Pontus Formation (Upper Miocene–Lower Pliocene) comprises lacustrine gravels, sands and clays passing laterally and upwards into fluvial sediments. The 300 m Upper Pontus Formation (Upper Pliocene–Lower Pleistocene) also consists of fluvio-lacustrine sediments but passes laterally into colluvium in the Cerkes region. Unlike the mainly marine Neogene successions bordering the Aegean, the minor divisions of the Pontus Formation have not been dated precisely; the continental sediments have yielded few fossils of biostratigraphical value.

Fig. 1 a, Plate setting of the studied segment of the North Anatolian transform fault zone (after ref. 2). b, Locations of the sampled Neogene basins. The traces of active fault breaks generated during six large magnitude earthquakes are also shown.

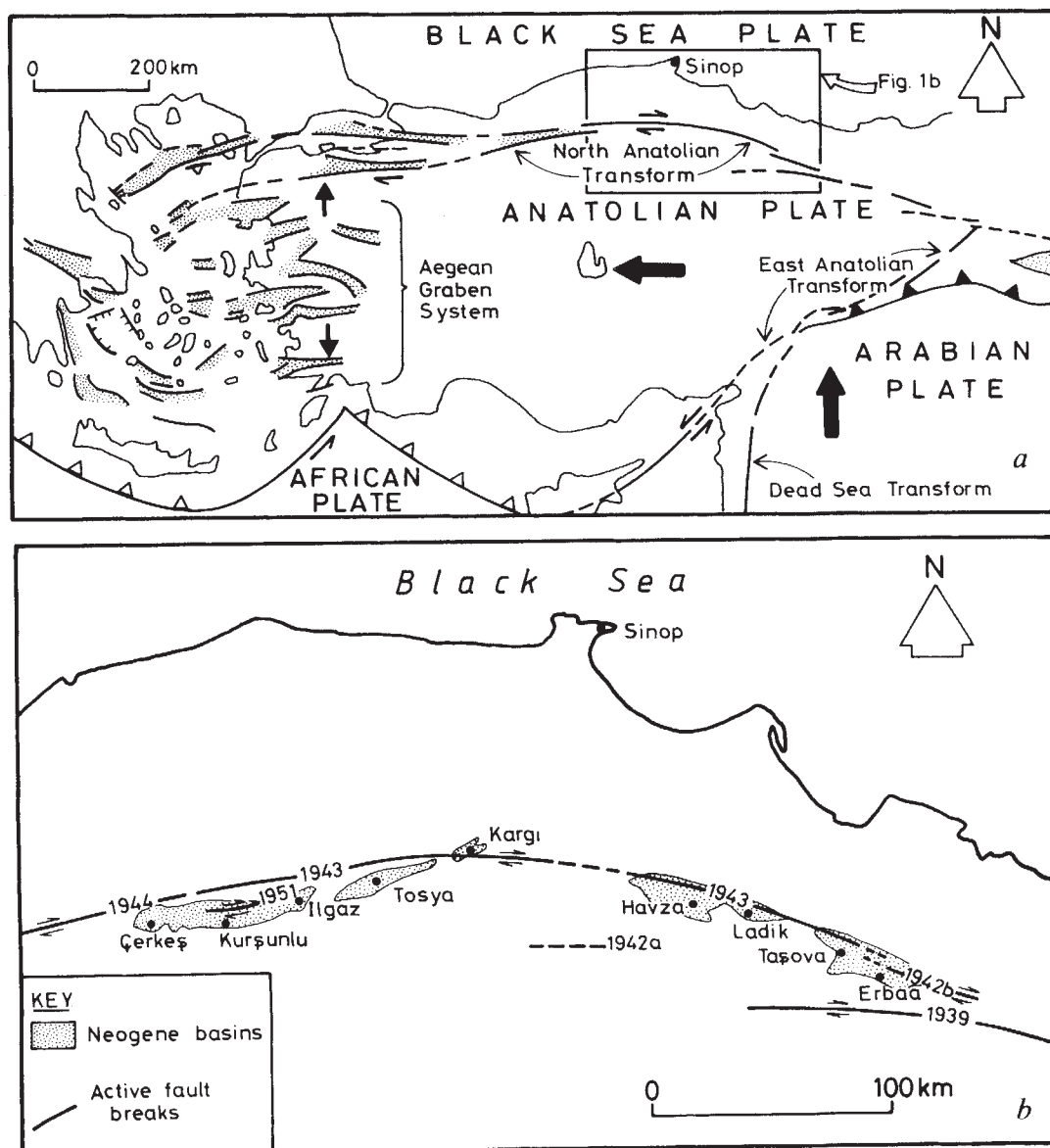


Table 1 Principal directions of compression or extension inferred from the analysed mesofractures

Basin		Reverse mesofaults		Normal mesofaults		Steeply inclined joints		Vertical joints		Trend of fault zone
		Analysed fractures	σ_1	Analysed fractures	σ_3	Analysed fractures	σ_3	Analysed fractures	σ_3	
Cerkas-Kursunlu-Ilgaz	West	Single fault set	087-267	Single fault set	320-140	Conjugate shear joints	351-171	Single set of extension joints	324-144	080
	Centre	Conjugate faults	065-245	Conjugate faults	330-150			Conjugate shear joints	343-163	078
	East	Single fault set	042-222	Conjugate faults	287-107	Conjugate shear joints	299-119	Conjugate shear joints	303-123	069
Tosya				Conjugate faults	318-138	Conjugate shear joints	312-132	Conjugate shear joints	331-151	079
Kargi				Conjugate faults	335-155	Conjugate shear joints	345-165	Conjugate shear joints	337-157	090
Havza-Ladik								Conjugate shear joints	336-156	110
Tasova-Erbaa				Conjugate faults	325-145			Conjugate shear joints	344-164	120
Number of analysed fractures		20		65		65		380		

σ_1 (maximum principal stress direction) and σ_3 (minimum principal stress direction) are given as horizontal projections of those directions.

Where growth faults and/or contemporaneous (intrasquence) erosion surfaces truncate fault planes, it is possible to distinguish between synsedimentary and post-sedimentary faults. However, because the movements responsible for generating the fractures were approximately identical during basin filling and later tectonism, the distinction between the two categories of faults is of less kinematic significance than in rocks where the time interval between deposition and subsequent tectonism is longer. Synsedimentary and post-sedimentary faults occur throughout the Pontus Formation and it is not possible to recognize horizons corresponding to particular tectonic events. Both faults and joints are less frequent in the Upper Pontus Formation.

The structures sampled during the full survey of mesoscopic-scale fractures are either joints (85%) or faults (15%). The latter are rarely greater than 20 m² in area and show displacements generally not exceeding 2 m. The orientations of 1941 mesofractures at 142 localities were measured. Twenty-seven per cent of these fractures are interpreted as being related to left-lateral shear. Figure 2 shows the morphology of some of them, of which 14% are faults and 86% are joints. At each sample locality, the sediments are horizontal or uniformly dipping and orientation data were collected from small, structurally homogeneous domains of 15 m² area. Because the majority of analysed faults cut horizontal sediments and show vertical offsets, and because the intersects between conjugate faults are

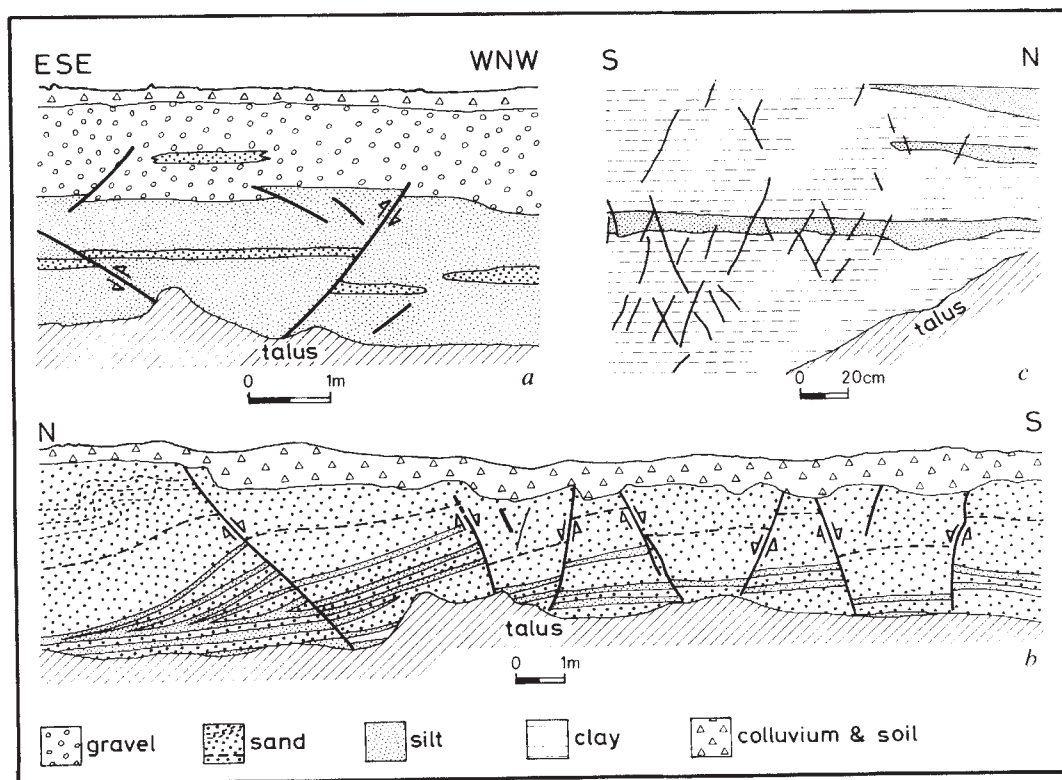


Fig. 2 Sketches from photographs of the analysed structures. *a*, Conjugate reverse mesofaults displacing gravels, sands and silts in the Lower Pontus Formation about 5 km west of Kursunlu. *b*, Conjugate normal mesofaults displacing cross-bedded sands and silts in the Lower Pontus Formation at Kursunlu. Synsedimentary folds are exposed at the north end of the section. *c*, Conjugate normal shear joints cutting silts interbedded with clays in the Lower Pontus Formation about 3 km south-east of Tosya.

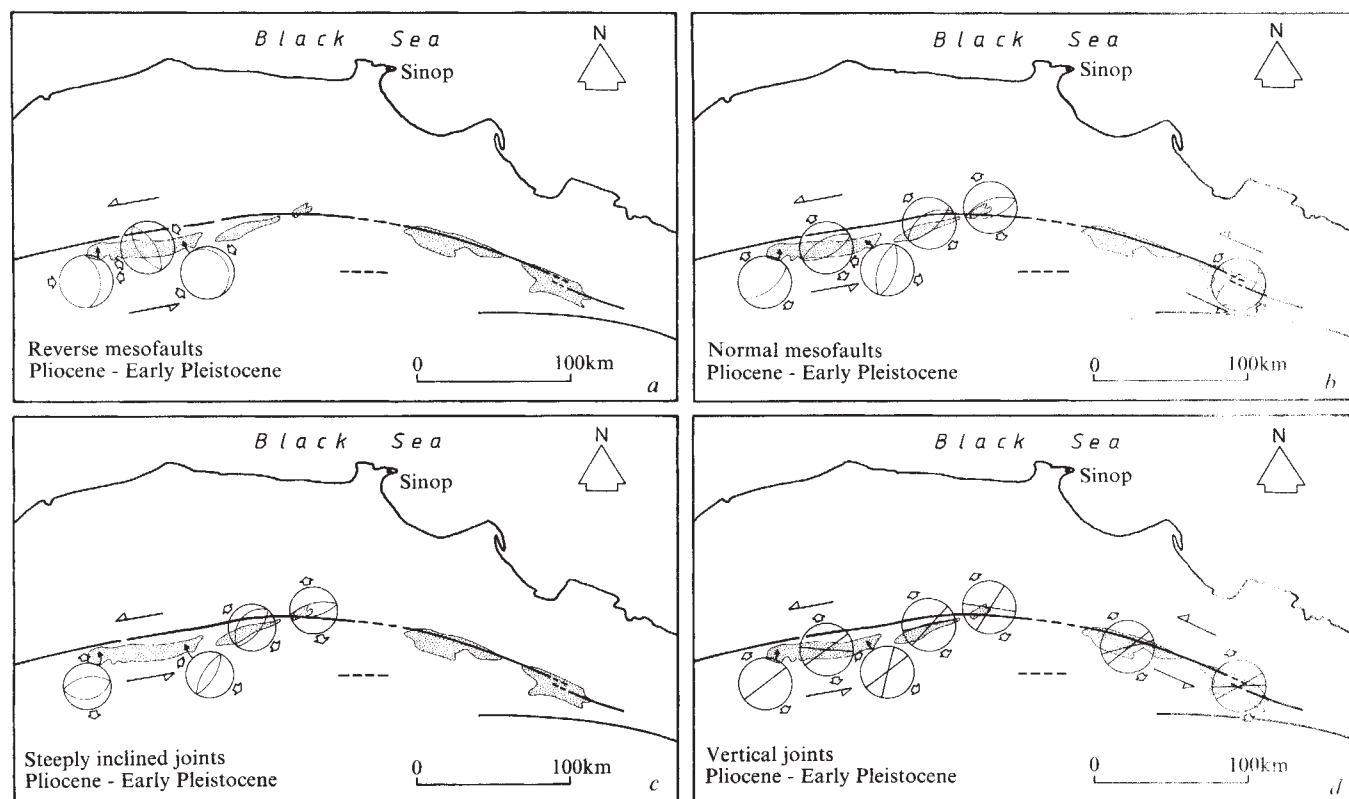


Fig. 3 Mean cyclographic traces of mesofracture sets of Pliocene-early Pleistocene age and from which a left-lateral sense of displacement on the North Anatolian fault zone may be inferred. Stereograms are lower-hemisphere Lambert plots of sets in either horizontal sediments or tilted sediments imagined to be restored to the horizontal. Pairs of arrows immediately outside each plot show horizontal directions of compression or extension inferred from the fractures, and believed to be related to the influence of left-lateral shear along the fault zone. See Fig. 1b for names of basins and dates of earthquake fault breaks. *a*, Reverse mesofaults. *b*, Normal mesofaults. *c*, Steeply inclined joints. *d*, Vertical joints.

horizontal or gently plunging, we conclude that displacements were dominantly dip-slip, either reverse or normal.

The fractures were analysed by determining the mean orientations of sets, field relationships being used to decide whether they are conjugate. Although conjugate faults are not present at all localities, it was possible to allocate single fault sets to an appropriate system by comparing their geometry with that of parallel joints belonging to conjugate sets. Approximate orientations of principal stress directions (Table 1) were determined from geometrical analysis of conjugate sets or single extension fracture sets. It was not possible to use the more precise technique of analysing striations on failure surfaces to determine either the orientations of principal strain axes⁹ or stress axes¹⁰ because only 11% of the faults are striated, and because many of the analysed structures are joints, which by definition cannot be lineated. It was unrealistic to determine the orientations of strain axes because, to get any regional meaning from them, an areal knowledge of the total slip on each set is required; unless the total slip on both conjugate sets is equal, the preferential development of one set rotates the *X* and *Z* axes. There was no evidence for more than one episode of displacement on any fault which is probably due to the mesoscale nature of the fractures. The determination of stress directions from conjugate shears is valid theoretically only when the fractures cut mechanically isotropic rocks. However, as field studies^{6,11} demonstrate, many rock masses contain numerous sets of symmetrically arranged mesofractures, and hence as Couples¹² states "... the ordered orientations of fractures indicate that the formation of one crack, and even the formation of many fractures, does not appreciably alter the orientation of the causative stresses, otherwise, the ordering would not be obtained".

The ~27% of mesofractures from which it is possible to infer that there was some left-lateral shear on the North Anatolian fault zone belong to four categories: reverse mesofaults; normal

mesofaults; steeply inclined joints; and vertical joints (Fig. 3). Whereas fractures related to right-lateral shear are abundant in both the Pontus Formation and the overlying Upper Pleistocene-Holocene sediments, those related to left-lateral shear are restricted to the Pontus Formation. The number of localities at which mesofractures related to different shear regimes intersect each other is limited. Most of those interpreted as being related to left-lateral shear are cut by those related to right-lateral shear.

North-west trending reverse mesofaults are the least abundant fractures (Fig. 3a), being observed only in the Cerkas-Kursunlu-Ilgaz basin where they are relatively common in the Lower Pontus Formation in the region of Kursunlu (Fig. 2a). Normal mesofaults (Figs 2b and 3b) strike between NNE and ENE. They are equally abundant in both the Upper and Lower Pontus Formations, but as Fig. 3b shows, they are restricted to the western basins with the exception of Tasova-Erbaa. Figure 3c shows the distribution of joint sets at mean inclinations between 50 and 74°. These fractures, which are commonly parallel or subparallel to nearby normal mesofaults, are interpreted as conjugate normal shear joints (Fig. 2c). Their distribution is restricted to the three western basins in which they are more abundant in the Lower Pontus Formation. Vertical joints are common in both divisions of the Pontus Formation in the three western basins, but are restricted to the Lower Pontus Formation in the two eastern basins. Except for the western subarea of the Cerkas-Kursunlu-Ilgaz basin, where the system is represented by a single set of extension fractures, the vertical joints form conjugate sets (Fig. 3d). They enclose a mean dihedral angle which is 41°, suggesting that they are mainly surfaces in the shear-extension fracture transition.

Although mesofractures in Neogene basins external to the fault zone were not sampled in detail, a reconnaissance survey suggests that they are significantly less abundant than within the basins associated with the fault zone. Thus, we believe that the

neotectonic mesofractures described above are restricted to the fault zone and are not part of a regional system.

The restriction of mesofractures related to left-lateral shear to sediments of the Pontus Formation, together with the observation that both synsedimentary and post-sedimentary structures are represented, suggests that the movements responsible for their generation occurred, possibly intermittently, during the late Miocene–early Pleistocene interval but did not continue after that. The greater abundance of the fractures in the west (79% of analysed surfaces) may indicate that left-lateral shear was more along the western part of the fault zone.

The difficulty of dating divisions of the Pontus Formation prevents the recognition of individual phases of compression or extension. The attitudes and displacement senses on the mesofractures are consistent with their development as secondary structures within a zone along which there was left-lateral displacement, a sense of shear which is the reverse of that along the North Anatolian fault zone at present.

A comprehensive explanation for the inference that there was some left-lateral shear on the fault zone is beyond the scope of this letter. Left-lateral shear may have been a consequence of either or both: a reversal of motion, possibly related to reversals detected in the Aegean region¹³; or the effect of local mechanical constraints caused by, for example, anti-Riedel¹⁴ overlaps of faults.

Fieldwork was funded by M.T.A. of Ankara and A.A.B. acknowledges that institute for a post-graduate award. We thank Nicholas Ambraseys for his comments about the North Anatolian fault zone and his advice. Jean Bees drafted the figures.

Received 28 February; accepted 12 June 1980.

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Sr isotopic geochemistry of voluminous rhyolitic ignimbrites and related rocks, Batopilas area, western Mexico

Marvin A. Lanphere*, K. L. Cameron†
& Maryellen Cameron†

* US Geological Survey, Menlo Park, California 94025

† Board of Earth Sciences, University of California, Santa Cruz, California 95064

The middle Tertiary ignimbrites of the Sierra Madre Occidental of western Mexico form the largest continuous rhyolitic province in the world. Minor amounts of intermediate lavas occur interlayered with the ignimbrites, and Sr isotope data on the complete range in rock compositions must be considered when evaluating hypotheses for the origin of the rhyolites. Initial ⁸⁷Sr/⁸⁶Sr ratios of andesites, dacites and rhyolites from the Batopilas region of western Chihuahua lie in the range 0.7042–0.7050, showing no systematic variation with rock composition. The data reported here support a crystal fractionation hypothesis for the origin for the voluminous rhyolitic ignimbrites.

A high volcanic plateau, the Sierra Madre Occidental, parallels the west coast of Mexico for 1,200 km (Fig. 1). The

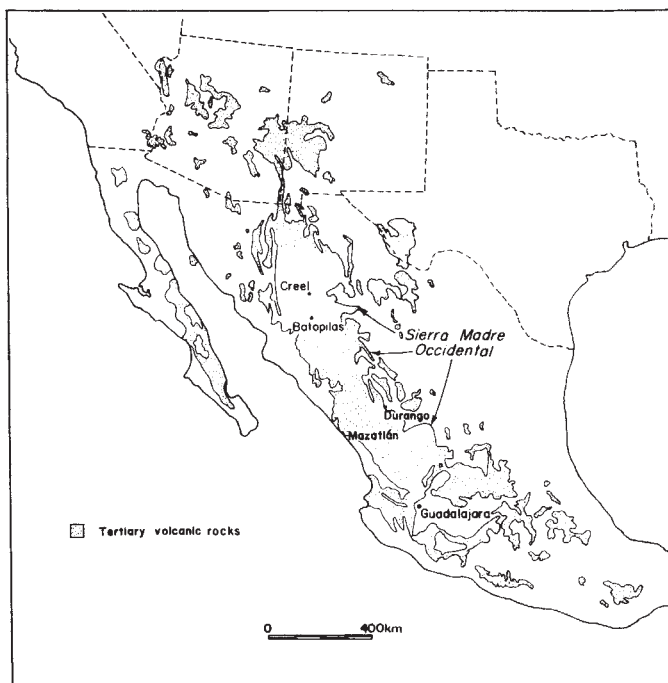


Fig. 1 Distribution of Tertiary volcanic rocks in Mexico and the adjacent US.

plateau is capped by a thick section of middle Tertiary extrusive rocks, predominantly rhyolitic ignimbrites (ash-flow tuffs), referred to informally here as the Upper Volcanic sequence. These ignimbrites, most of which were erupted between 34 and 27 Myr ago, cover at least 200,000 km² in western Mexico and have an average thickness^{1–3} approaching 1,000 m. An eastward-dipping subduction zone was active off the west coast of Mexico when these rocks were erupted⁴. The volcanic deposits occur as a belt parallel to the western continental margin (Fig. 1), suggesting that they represent a middle Tertiary magmatic arc associated with that subduction⁵. The Sierra Madre Occidental contains a larger volume of rhyolitic rocks than do better known ignimbrite provinces such as the Central Andes (>70,000 km³, ref. 6), North Island, New Zealand (>15,000 km³, ref. 7) and Central America (>10,000 km³, ref. 8). The total original volume of rhyolitic ignimbrites in the western US may have exceeded that of Mexico, but much of that has now been removed by erosion or covered by more recent deposits.

Most of the samples described here are from the Batopilas region of western Chihuahua (Fig. 1). Data on a few samples of rhyolite from the Creel area (Fig. 1), about 100 km north of Batopilas, are also reported. The Upper Volcanic sequence near Batopilas was erupted ~30 Myr ago³. More than 80% of the sequence is composed of rhyolitic ignimbrites, but rhyolitic, dacitic and andesitic lava flows occur interlayered with the ignimbrites. The andesite-to-rhyolite series is calc-alkalic and contains ~1% K₂O at SiO₂ = 60% (refs 3, 5); the ignimbrites typically contain 71–75% SiO₂ and 0.5–1.5% normative corundum. The present study compared the Sr isotopic compositions of the voluminous rhyolites with those of the associated intermediate lavas. The only previously published Sr isotopic data on rocks of the Upper Volcanic sequence were on eight samples of rhyolite collected along a transect between Durango and Mazatlán (Fig. 1), some 400 km south of the Batopilas area, and three samples from near Cuauhtemoc, ~175 km north-east of Batopilas⁹. The initial ⁸⁷Sr/⁸⁶Sr ratios of these samples range from 0.7033 to 0.7062.

Rb and Sr concentrations were determined by X-ray fluorescence. The uncertainties in concentration measurements are estimated to be ~3% based on replicate analyses of USGS rock standards. Sr isotopic composition was measured on samples dissolved in HF and HClO₄; Sr was separated using ion-

Table 1 Rb, Sr and Sr isotopic compositions of samples from the Upper Volcanic sequence of the Sierra Madre Occidental, western Chihuahua, Mexico

Sample no.	Rock type or mineral	SiO ₂ (wt %)	Rb* (p.p.m.)	Sr* (p.p.m.)	⁸⁷ Rb/ ⁸⁶ Sr	(⁸⁷ Sr/ ⁸⁶ Sr) [†] Measured	(⁸⁷ Sr/ ⁸⁶ Sr) [‡] Initial
Batopilas area							
MX-7526	Lava	60.5	72	437	0.476	0.70491 ± 17	0.70471 ± 17
MXU-7616	Lava	61.0	86	468	0.531	0.70497 ± 17	0.70474 ± 17
MX-7528	Lava	62.4	65	392	0.480	0.70468 ± 20	0.70448 ± 20
MXB-7714	Lava	64.5	244	418	1.69	0.70538 ± 19	0.70466 ± 19
MX-7618	Lava	65.2	79	402	0.568	0.70495 ± 9	0.70471 ± 9
MX-7530	Lava	67.6	136	319	1.23	0.7053 ± 3	0.7048 ± 3
MX-7530	Plagioclase	—	—	—	—	0.70485 ± 25	—
M-172	Ignimbrite§	71.5	139	373	1.08	0.70545 ± 20	0.70499 ± 20
MXU-7615	Ignimbrite	72.1	133	196	1.96	0.70519 ± 14	0.70435 ± 14
MX-7522	Ignimbrite	73.5	87	252	0.999	0.7052 ± 2	0.7048 ± 2
MX-7522	Plagioclase	—	—	—	—	0.70475 ± 20	—
M-161	Ignimbrite	74.7	179	160	3.24	0.70623 ± 16	0.70485 ± 16
M-220	Lava	74.9	114	164	2.01	0.70505 ± 29	0.70419 ± 29
MX-7617	Lava	75.4	133	143	2.69	0.70563 ± 17	0.70448 ± 17
MX-7504	Ignimbrite	76.2	267	94	8.22	0.7081 ± 3.5	0.7046 ± 3.5
MX-7504	Plagioclase	—	—	—	—	0.7051 ± 2.5	—
Creel area							
M-205	Lava	73.0	164	295	1.61	0.70812 ± 10	0.70743 ± 10
M-276	Lava	76.2	170	197	2.50	0.70660 ± 11	0.70554 ± 21
M-103	Lava	76.3	118	157	2.17	0.7071 ± 2	0.7063 ± 2

* Rb and Sr concentrations measured by X-ray fluorescence.

† ⁸⁶Sr/⁸⁸Sr ratios normalized to a value of 0.1194. ⁸⁷Sr/⁸⁶Sr ratios adjusted to a value of 0.71014 for NBS SrCO₃ standard. Analyses were made at US Geological Survey, Menlo Park.‡ Corrections to measured ⁸⁷Sr/⁸⁶Sr ratios due to addition of radiogenic ⁸⁷Sr are based on K–Ar ages, ref. 3.

§ All ignimbrites are whole rock samples.

|| 40 km south of Creel.

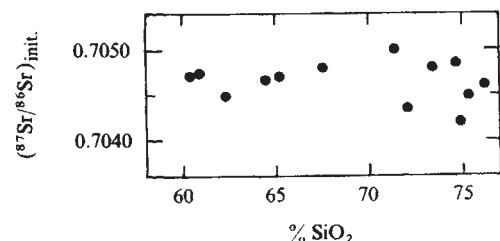
exchange chromatography. Mass spectrometry was done with an automated 30.48-cm-radius 90°-sector single-collector instrument using a triple-filament source and digital data acquisition. A typical Sr-composition determination consists of 24–36 measurements of each mass. The uncertainties in isotopic composition (Table 1) are standard deviations of analytical precision.

The initial ⁸⁷Sr/⁸⁶Sr ratios for 13 samples of andesitic to rhyolitic rocks of the Upper Volcanic sequence from the Batopilas area range from 0.7042 to 0.7050 (Table 1). There is no systematic variation in initial ratios with SiO₂ content (Fig. 2), Sr content or Rb/Sr ratio. The Pearson correlation coefficient, *r*, between the initial ⁸⁷Sr/⁸⁶Sr ratio and SiO₂ content, Rb content, and Rb/Sr ratio are –0.20, 0.05, and –0.15, respectively. The correlation coefficient between initial ⁸⁷Sr/⁸⁶Sr ratio and Sr content is 0.40 (Fig. 3). However, the null hypothesis that these two variables are linearly related is rejected at the 5% level of significance. That is, there is no significant correlation between the ⁸⁷Sr/⁸⁶Sr ratio and the Sr content of the analysed samples. Upper Volcanic rhyolites from Creel are more radiogenic than those from Batopilas, with initial ratios ranging from 0.7055 to 0.7074 (Table 1). Initial ratios reported by McDowell *et al.*⁹ for eight samples of rhyolite from the Durango-Mazatlan area range from 0.7033 to 0.7062.

Rocks of the Upper Volcanic sequence from the Sierra Madre Occidental have somewhat higher ⁸⁷Sr/⁸⁶Sr ratios than Quaternary andesites of the Trans-Mexico volcanic belt (0.7036–0.7043, ref. 10). The ignimbrites from Mexico are similar in Sr isotopic characteristics to Cenozoic ignimbrites from Nevada¹¹ and Central America¹² but are less radiogenic than ignimbrites from Chile¹³. The middle Tertiary andesite-to-rhyolite series of the San Juan Mountains volcanic field of Colorado, shows an increase in initial ⁸⁷Sr/⁸⁶Sr ratio from about 0.7054 to 0.7070 with increasing SiO₂ content¹⁴. This increase was interpreted¹⁴ to reflect interaction of the silicic magmas with Precambrian upper crust enriched in radiogenic Sr. In contrast to the San Juan ignimbrites, there is no evidence in the Sr isotopic data that the Batopilas rhyolites interacted with radiogenic crust. The nature of the crust beneath the region is uncertain, but Anderson and

Silver¹⁵ reported that the southern limit of the Precambrian craton probably lies about 200 km to the north. McKee *et al.*¹⁶ reached a similar conclusion for the origin of early Miocene (21–27 Myr old) rhyolitic ash-flow tuffs in the northwestern part of the Great Basin, US. Initial ⁸⁷Sr/⁸⁶Sr ratios ranging from 0.7042 to 0.7062 were interpreted to reflect only small amounts of contamination of mantle-derived magmas by crustal material.

The Sr isotopic data reported here support the fractional crystallization model proposed recently by Cameron *et al.*¹⁷ for the origin of the Batopilas rhyolites. The rare-earth element patterns of the ignimbrites can be modelled closely using associated andesites and dacites as parents (K.L.C. and G. N. Hanson, unpublished data). The fractionating assemblage was dominated by plagioclase (>60%) and contained ~10% magnetite with the remaining phases being pyroxene and/or hornblende. The ultimate parent was probably a basaltic magma of mantle origin. Th–Sr variations within the andesite to rhyolite series are inconsistent with a hypothesis that the series were derived by different degrees of melting of similar crustal sources (M.C., K.L.C. and W. C. Bagby, unpublished data). The rhyolites of the Sierra Madre Occidental are heterogeneous isotopically as shown by a comparison of the Batopilas and Creel data (Table 1) and the analyses reported by McDowell *et al.*⁹. Some of the silicic magmas may have interacted with radiogenic

**Fig. 2** Initial ⁸⁷Sr/⁸⁶Sr ratio plotted against SiO₂ content for rocks in the Upper Volcanic sequence of the Batopilas area.

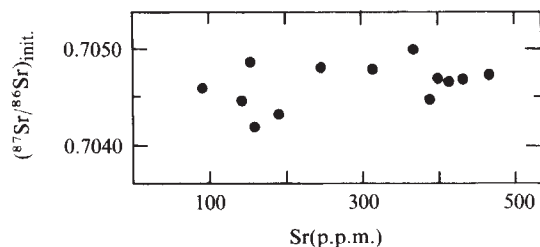


Fig. 3 Initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio plotted against Sr content for rocks in the Upper Volcanic sequence of the Batopilas area.

crust. Nevertheless, the Sr isotope data from the Batopilas region establish that no such interaction was required to produce the voluminous rhyolitic ignimbrites of the Sierra Madre Occidental.

Fieldwork was supported by NSF Grant EAR 77-09006. We also acknowledge partial support from the Donors of the Petroleum Research Fund, administered by the American Chemical Society. We thank W. C. Bagby for several of the samples, and A. L. Berry for making the Sr isotopic measurements at the laboratories of the US Geological Survey in Menlo Park.

Received 29 February; accepted 28 May 1980.

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Ancient Archaean supracrustal rocks from the Limpopo Mobile Belt

Peter C. Horrocks

Department of Geology, University of the Witwatersrand, Jan Smuts Avenue, Johannesburg 2001, South Africa

A preliminary study has been made to establish pressure, temperature and water activity parameters for the high-grade metamorphism of the early Archaean supracrustal rocks exposed in the Limpopo Mobile Belt near Messina, South Africa. The results reported here indicate that a significant orogenic crustal thickness up to ~30 km was developed before ~3,100 Myr ago in the region.

The high-grade and poly-deformed supracrustal gneisses studied occur south-east of Messina in the Central Zone¹ of the Limpopo Mobile Belt (Fig. 1). They are thought to have been deposited at least partly on a sialic basement made up of the ~3,800 Myr old Sand River Gneisses², which are cut by 3,570 Myr old tholeiitic dykes² that have not been recognized in the supracrustal gneisses. The supracrustal gneisses were intruded by the anorthositic, gabbroic and peridotitic gneisses of

the Messina layered intrusion³ probably ~3,270 Myr ago (J. M. Barton Jr, work in preparation). Subsequently, an important event of igneous and metamorphic activity affected all of these rocks ~3,150 Myr ago⁴, and a second suite of ~3,060 Myr old tholeiitic dykes were intruded².

The supracrustal gneisses are predominantly quartz-feldspathic, but contain numerous interlayers and lenses of pyroxenitic amphibolite, quartzite, magnetite quartzite, garnet-cordierite gneiss, calc-silicate gneiss and marble. The mafic lithologies commonly preserve orthopyroxene + clinopyroxene + amphibole + plagioclase + quartz + magnetite ± garnet assemblages, and frequently show prograde metamorphic textures in which orthopyroxene has replaced and overgrown amphibole, while some localities show coexisting clinopyroxene and garnet. Kelyphitic coronas predominately made up of plagioclase are developed around the garnet in lithologies with abundant amphibole, and in some samples, the garnet is almost completely replaced, or remains only as a few scattered optically continuous remnants in the centres of these reaction coronas. Thus, these kelyphitic textures are thought to be the result of retrograde processes (ref. 5, and P.C.H., in preparation), and not due to a second prograde metamorphism as has been described for previously metamorphosed rocks with a resulting low water partial pressure⁶. The metapelitic lithologies contain garnet + cordierite + biotite + sillimanite (± kyanite) + plagioclase + quartz ± K-feldspar ± amphibole ± orthopyroxene. The garnet is commonly distinctly zoned with more inclusion-filled cores, which are more pyrope-rich, overgrown by thin more almandine-rich and inclusion-poor rims (work in preparation). Some localities preserve kyanite overgrown by sillimanite⁷⁻⁹.

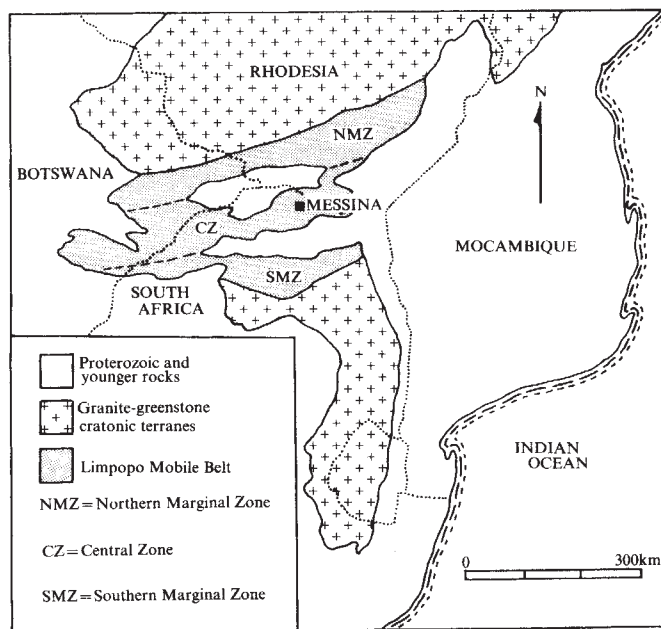


Fig. 1 A simplified geological map of part of southern Africa showing the location of Messina and the Limpopo Mobile Belt.

Peraluminous and magnesia-rich rocks within these metapelitic gneisses consist of corundum + spinel + sapphirine + cordierite + garnet + phlogopite ± orthopyroxene ± gedrite, and these undersaturated assemblages apparently represent metamorphic residua after the extraction of minimum melt granitic anatectic liquids^{10,11}. Symplectitic coronas about garnet are developed in places where the garnet is replaced by an intergrowth of radial orthopyroxene grains in plagioclase (Fig. 2). This garnet is relatively unzoned, and it is not obvious whether this reaction is prograde or retrograde. However, some samples preserve only spherical 'knots' comprising myrmekitic orthopyroxene grains in plagioclase suggesting the complete breakdown and

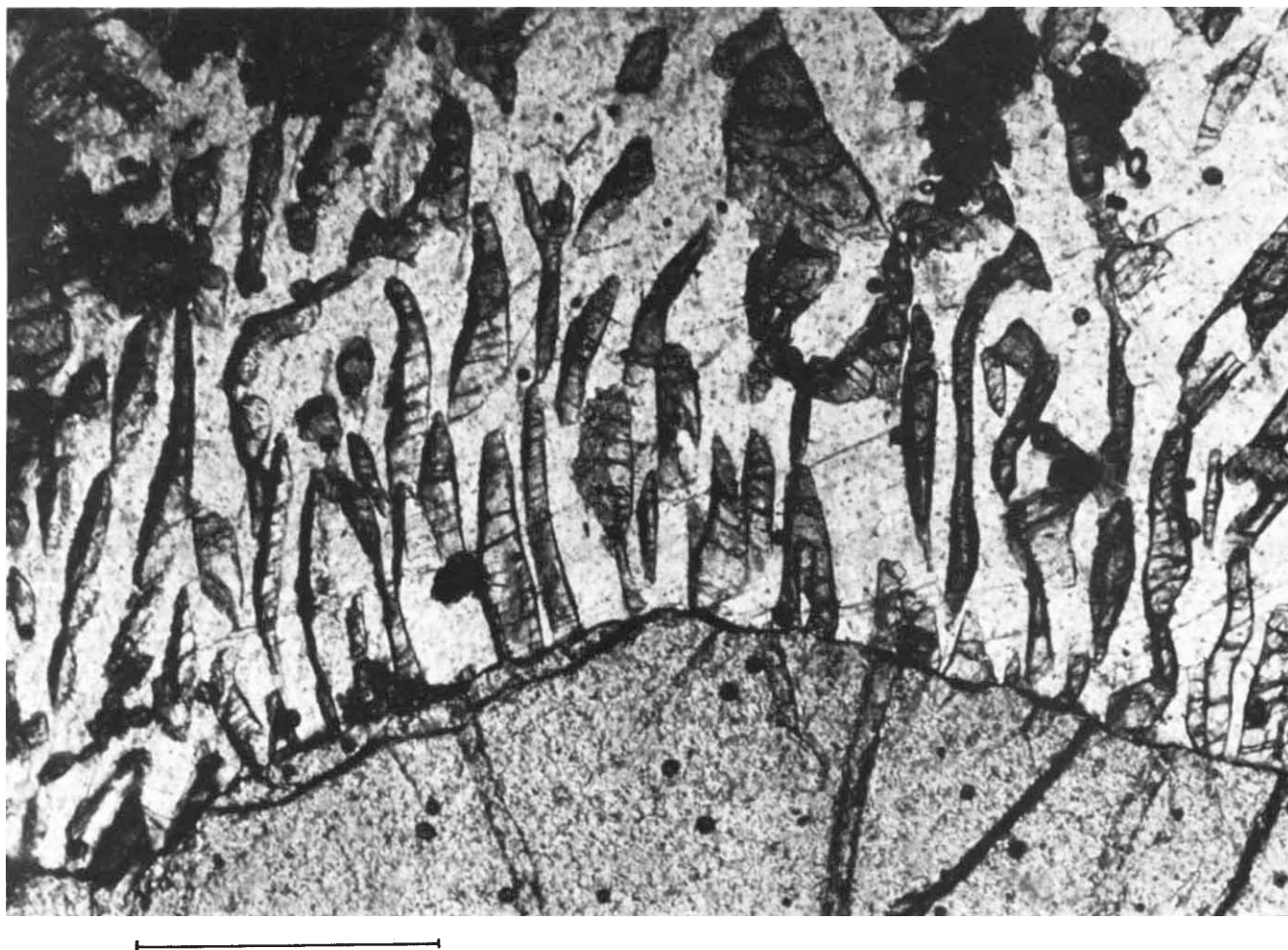


Fig. 2 A photomicrograph of the garnet-orthopyroxene-plagioclase symplectite. Note the worm-like orthopyroxene grains in the plagioclase which radiate out from the garnet porphyroblast. Scale bar, 0.5 mm.

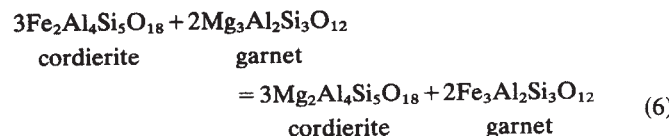
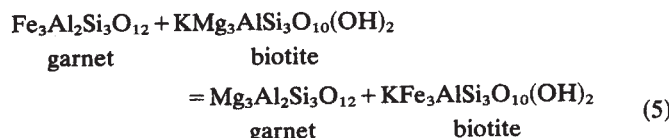
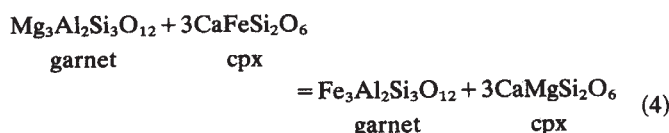
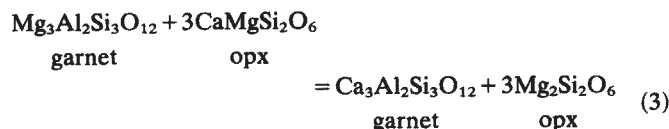
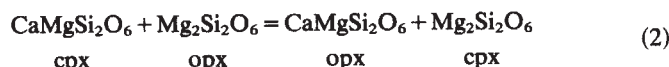
Table 1 Temperature, pressure and water activity estimates for the supracrustal rocks near Messina, Limpopo Belt

Equilibria Coexisting minerals	(1) & (2) opx + cpx	(3) opx + gar-rim	(4) cpx + gar-rim	(5) gar-rim + biot	(6) gar-rim + cord	(7) opx + cpx + hble + qz
Refs	12-14	12, 14, 16, 21	15-17	18, 19	16, 18	23
Mafic rock samples						
27	890 ± 50	660 ± 50 6.3 ± 3	690 ± 50 at 6 kbar	—	—	0.10 ± 0.01
X21395	870 ± 50	—	—	—	—	0.06 ± 0.01
X21399	870 ± 50	665 ± 50 5.5 ± 3	690 ± 50 at 6 kbar	—	—	0.03 ± 0.01
Pelitic samples						
21-7-F	—	—	—	755 ± 50 at 6 kbar	630 ± 50 7 ± 1.5	—
21-7-G	—	—	—	730 ± 50 at 6 kbar	750 ± 50 6.5 ± 1.5	—
Symplectite						
2-8-10B	—	680 ± 50 5.6 ± 3	—	640 ± 50 at 6 kbar	—	—
Sapphirine rocks						
2-8-12	—	—	—	640 ± 50 at 6 kbar	610 ± 50 8.1 ± 1.5	—
11-8	—	780 ± 50 at 6 kbar	—	655 ± 50 at 6 kbar	590 ± 50 8.2 ± 1.5	—

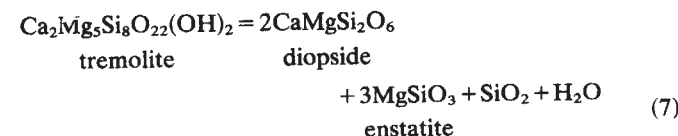
See text for details of the equilibria used, and a discussion on the errors. Temperatures are in °C; pressures in kbar; water activities are expressed as a fraction between 0 and 1 for equilibrium 7 only. biot, biotite; cord, cordierite; cpx, clinopyroxene; gar, garnet; hble, hornblende; opx, orthopyroxene; qz, quartz.

replacement of garnet probably by a retrograde process similar to that which is thought to have produced the kelyphitic coronas.

Electron microprobe analyses of the above mineral assemblages mean that several thermodynamic techniques can be used to estimate some of the pressure, temperature and water activity parameters of the high-grade metamorphism these rocks have experienced. The analytical technique used natural and synthetic standards, and was routinely able to repeat known international standards typically to within 2% for all relevant elements. Temperature estimates have been calculated using the following equilibria:



The geothermometers derived from these equilibria¹²⁻¹⁹ are based on experimental data and calculated values are typically within 50 °C of the experimental condition. Thus the maximum practical resolution of these techniques is probably about ±50 °C and about ±100 °C at worst. Fe³⁺ was estimated in pyroxene and garnet by a stoichiometric method²⁰ and was found to cause generally a lowering of the temperature estimates by not more than 50 °C in most cases, and usually much less than 50 °C. Pressure estimates were made from equilibria (3) and (6), and these geobarometers^{12,16,21}, also based on experimental data, have given more inconsistent results²², mainly due to the problems surrounding Al solution in pyroxene. At worst, these barometers reproduce the experimental values to within 5 kbar (ref. 12) and to within 1.5 kbar (ref. 16) for the garnet–cordierite equilibrium. Typically, the best resolution for practical purposes is probably limited to ±3 kbar (ref. 21). The water activity was calculated using equilibrium (7) (S. Richardson, personal communication) in the mafic lithologies:



together with certain thermodynamic approximations²³ based on hydrothermal equilibrium data. The results from these calculations are summarized in Table 1 and Fig. 3.

The *P*–*T* field thus suggested for the supracrustal rocks in the region (Fig. 3) supports the petrographical evidence of this study and that of other studies in the region⁷⁻⁹, particularly with regard to the association of enstatite + kyanite + quartz⁷, sillimanite overgrowths on kyanite⁷⁻⁹, prograde orthopyroxene replacement of amphibole (that is, granulite facies metamorphism), retro-

grade kelyphitic coronas about garnet in the mafic rocks, and the garnet zonation in the pelitic rocks. The early phase of this metamorphism which produced the orthopyroxene-bearing assemblages in the mafic rocks, and the pyrope-rich garnet and corundum in the pelitic rocks (work in preparation), was characterized by temperatures in excess of 800 °C and probably pressures up to 10 kbar (Fig. 3). Water activities in the mafic rocks were low, with H₂O making up ≤10% of the fluid present during the high-grade phase of the metamorphism. CO₂ was probably the most abundant fluid present during this time, based on a comparison with a study of liquid inclusions in granulites from southern Norway²⁴. A subsequent retrogression to a lower temperature–pressure regime is recorded by the development of the kelyphitic rims about garnet in the mafic lithologies, the almandine-rich garnet overgrowths and the spinel + sapphirine + cordierite association (work in preparation) in the pelitic rocks, and probably also the production of the symplectitic textures about garnet (Fig. 2). Here, pressures of between 5 and 7 kbar were attained at temperatures of ~600–700 °C (Fig. 3).

The early phase of this metamorphism suggests that the supracrustal stratigraphy, deposited sometime between 3,570–Myr ago and the emplacement of the Messina layered intrusion ~3,270 Myr ago, was subjected to rapid burial to depths probably greater than 30 km into regions of the lower crust either by subsidence or tectonic means. An earlier study of an enstatite + kyanite + quartz association from Zimbabwe⁷ also suggested pressures in excess of 10 kbar and temperatures close to 950 °C with the enstatite having an Al₂O₃ content of over 5.5%. These data suggest that this metamorphism reflects an average geothermal gradient during the peak of the metamorphic event of less than about 30 °C km⁻¹ in the lower regions of the crust. This compares favourably with other suggested Archaean geotherms²⁵⁻²⁸. The metamorphic and igneous activity about 3,150 Myr ago, which reset the Rb–Sr isotopic ratios in the rocks of the Messina layered intrusion³ probably reflects the peak of this phase of the metamorphism. Subsequent orogenic events occurred during the interval from ~3,100 Myr ago to ~2,400 Myr ago⁴ during which minimum melt granitic anatectic liquids were extracted²⁹ and intense polyphase deformation was imposed on the lithologies⁹. Although temperature and pressure data currently available do not provide sufficient evidence in themselves, these events could probably represent a continuous progression of tectonism and metamorphism^{8,9} during the

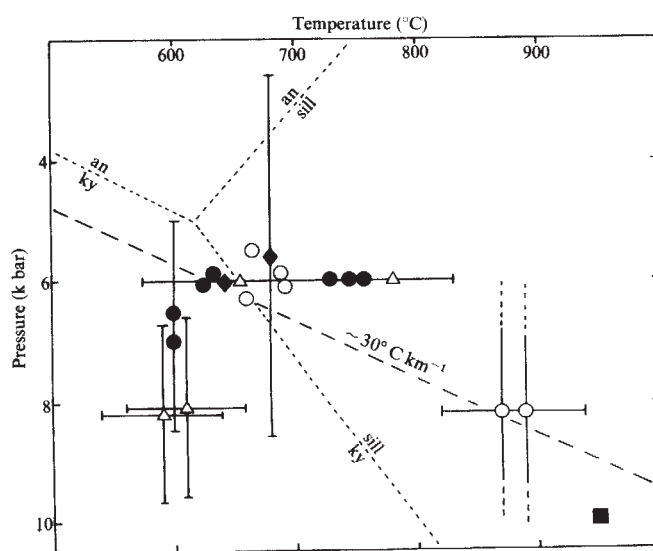


Fig. 3 A pressure–temperature diagram showing the results given in Table 1: ○, mafic rocks; ●, pelitic lithologies; △, sapphirine-bearing samples; ◆, symplectite sample; ■, enstatite + kyanite + quartz⁷ association discussed in the text. an, andalusite; ky, kyanite; and sill, sillimanite.

erosional and/or tectonic excavation of the supracrustal rocks and the resulting pressure and temperature changes. The exhumation of these gneisses would cause waning pressures at maintained temperatures, probably adiabatically, and may explain the retrogression as manifest by the kelyphitic textures and garnet zonation. The end of high-grade metamorphism and the onset of comparative tectonic stability are evidenced by a fabric-free dolerite dyke of ~2,200 Myr in age³⁰. This ancient basement-supracrustal event of high-grade metamorphism may not predate the development of the greenstone-grandodiorite-tonalite terrains which comprise much of the Earth's shield

areas, but perhaps represents a contemporaneous development at lower crustal levels now only exposed in limited zones of the ancient shields. The geometry of these zones (or 'belts') is probably superimposed, and not a result of the intrinsic metamorphic history.

This work was sponsored by the South African Council for Scientific and Industrial Research, and is Contribution No. 56 in South Africa's contribution to the International Geodynamics Project. I thank T. N. Clifford, W. Schreyer, A. B. Thompson, D. J. Ellis, R. E. P. Fripp, J. M. Barton Jr and M. K. Watkeys for criticisms.

Received 12 February; accepted 3 June 1980.

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Evidence for pre-Devensian glaciation in the northeastern Lake District

J. Boardman

Department of Humanities, Brighton Polytechnic, Falmer, Brighton, East Sussex BN1 9PH, UK

In the northeastern English Lake District two major till units occur. The upper unweathered unit is clearly the product of late-Devensian glaciation¹. The lower till has undergone severe humid temperate chemical weathering and contains palaeosol features. It must pre-date the Ipswichian interglacial². Pre-Devensian weathering profiles of an interglacial character have not previously been described in northern England. There has been scant previous evidence for a pre-Devensian glaciation of north-west England^{3,4}. No date has been assigned to 'Early Scottish' till which has been located around Carlisle. Till in Low Furness is recorded beneath 'interglacial beds', but this was described in the nineteenth century and cannot be relocated^{4,5}. It has generally been accepted that erosion during the Devensian effectively removed pre-existing deposits and it has been assumed that the landforms of the Lake District, including the major erosional features, are the product of the last glaciation^{5,6}. I show here that, in the Vale of Threlkeld, this is not the case.

In the valleys of Mosedale and Thornsill Beck, two south bank tributaries of the Glenderamackin (Fig. 1), 10 km east of Keswick, a sequence of glacial, glacialfluvial and periglacial deposits overlie thinly-cleaved mudstones of Ordovician Skiddaw Group⁷. The surface tills form drumlins and till hummocks, and glacialfluvial sands and gravels locally form kames. All these features are considered to be of late Devensian age¹. The Mosedale Beck valley was eroded by high meltwater discharges released by the corrie glacier at Wolf Crag which last developed in Younger Dryas time⁴. In places, such as on Threlkeld Common, stone-banked solifluction lobes disturb the till and these are probably of Younger Dryas age.

The recently discovered succession in the area² reveals weathered till at the base, overlain by fresh unweathered till or unweathered sands and gravels. Four representative sites are considered in Fig. 2.

At Caryl Gully and site D, yellowish-brown weathered till is overlain by a grey till which incorporates, at its base, lenses of the weathered material. At two sites, D and N, a weathering profile exists—the severely weathered till grading down into fresh till. In the weathered till manganese staining is frequent, clasts are soft and friable, and surface textures on quartz grains, examined by scanning electron microscopy, indicate severe chemical weathering and an absence of glacial processes (P. Wilson, personal communication). At site E, the weathered till unit is dissected by a channel which is filled with similarly weathered sands and gravels. This in turn is overlain by coarse unweathered gravels which form a terrace fragment of Younger Dryas age.

At N, the upper part of the site is obscured but it is probable that unweathered Devensian material completes the sequence, as it is exposed at nearby sites. In places, a gleyed horizon is developed in a silty clay band at the base of the severely weathered till. The dominant colour is orange (7.5YR 6/8) but it contains many irregular mottles of low chroma (5Y 7/1). In thin section these are seen to be iron-depleted areas (albans). Channels and chambers reflect biological activity⁸, and in some cases, are lined with translocated clay and silty clay. Gleying, translocation of clay, and biological activity are characteristic of the B horizon of certain soils.

The chief characteristics of the weathered till in the area are: (1) high proportion of soft and friable clasts that can be crushed by hand; (2) typical bright yellowish-brown (10YR 6/6) clast colour and yellowish-brown (10YR 5/8) matrix colour which differ from greenish-grey (7.5GY 5/1) and dark grey (N 3/0) respectively of the fresh materials; (3) low silt and clay percentages, 13 and 7% for the weathered till samples, in contrast to values of 23 and 20% for unweathered equivalents; (4) clay mineral suites dominated by illite and vermiculite which are typical of many weathering products of acid igneous rock types (R. Merriman, personal communication).

The weathering profile is interpreted as having formed in a period of protracted weathering on a stable land surface such as is typical of a vegetated temperate interglacial environment.

The sequence of events can be summarized as: (1) deposition of the lower till and glacialfluvial sands and gravels; (2) severe chemical weathering under humid temperate conditions; (3) deposition of the upper till incorporating lenses of the pre-existing weathered till unit; (4) deposition of terrace gravels in Mosedale and corrie glaciation at Wolf Crag.

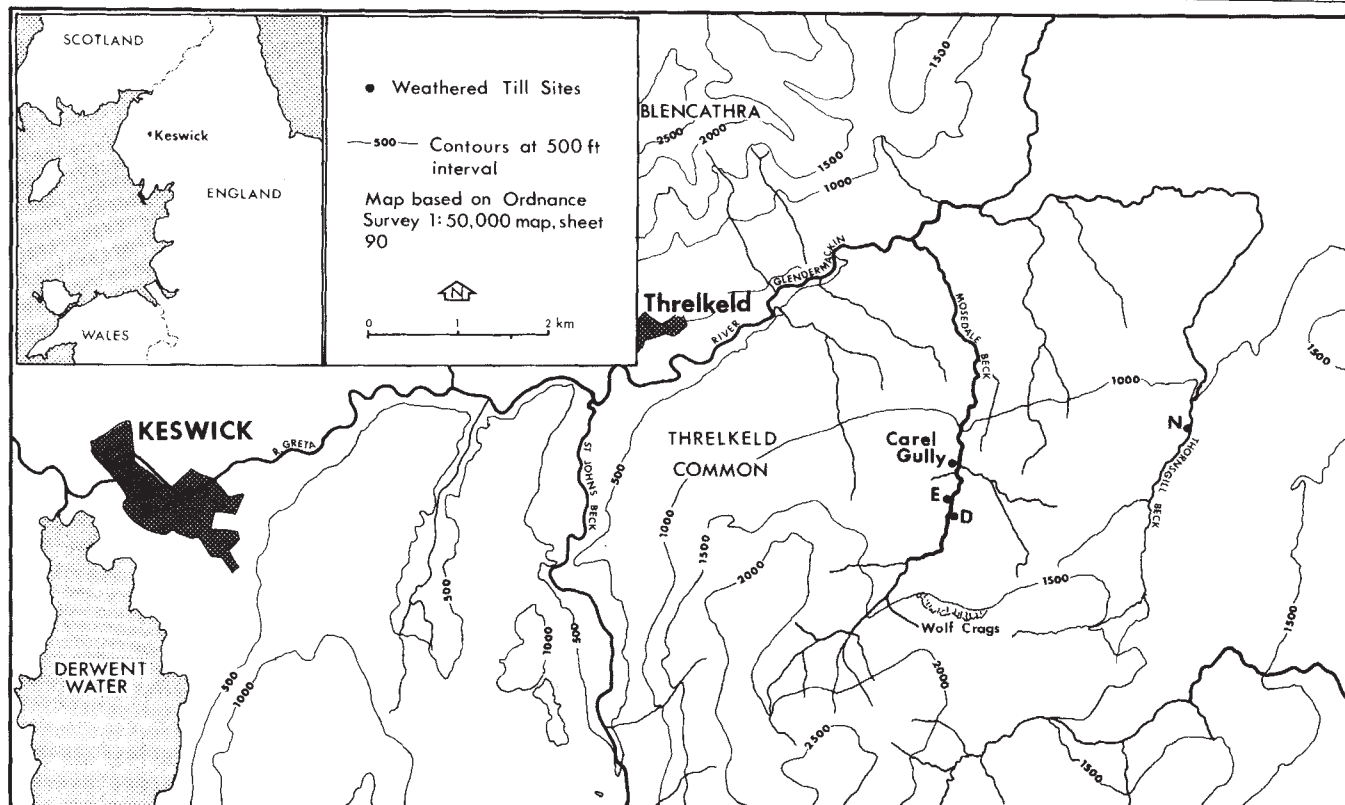


Fig. 1 Location of sites.

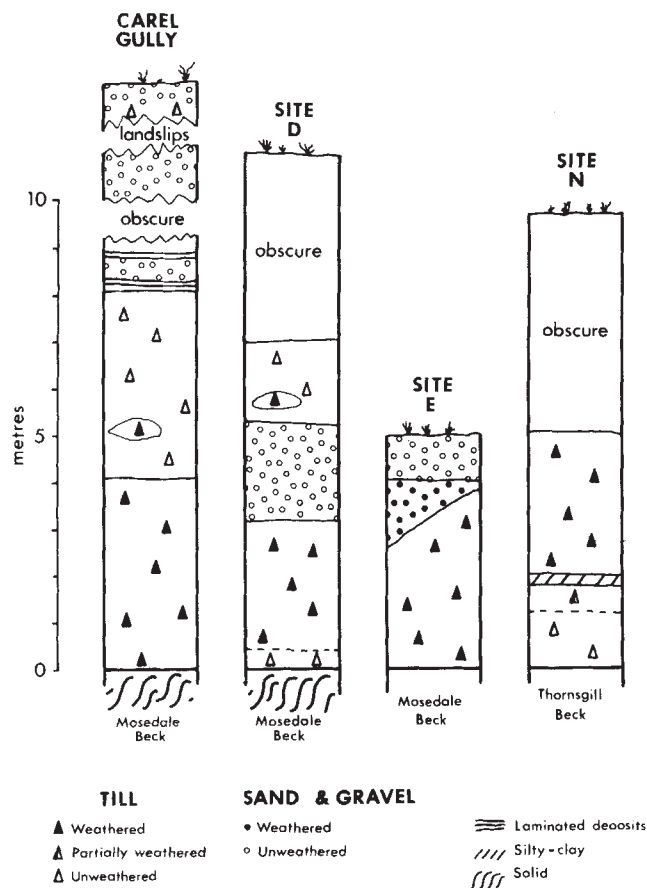


Fig. 2 Stratigraphy of sites.

With reference to the established British Quaternary stratigraphy⁹, the upper till and associated glacial landforms is Devensian, the weathering profile is most likely to be Ipswichian, and the weathered till unit is probably Wolstonian. Till of that age has been described from East Yorkshire and Lincolnshire¹⁰, and a possible Wolstonian till containing Lake District erratics occurs in the Peak District¹¹. However, in the Lake District, a maximum age cannot be assigned to the till: it may be of greater antiquity.

Preservation of unconsolidated sediments beneath till is rare in highland Britain, and in the northeastern Lake District seems to be the result of irregular bedrock topography. The survival of deposits at several sites suggests that Devensian erosion had a limited impact on the Vale of Threlkeld. Classic glacial features in the area such as the truncated spurs of Blencathra (Fig. 1) are therefore likely to be pre-Devensian in age. By implication, other major glacial troughs in the Lake District may also have been in existence at the onset of the last glaciation.

I thank Dr P. Bullock for advice on pedological aspects and Dr J. Catt for comments, Mr J. Rose, who discovered the weathered till in Mosedale, for discussions and comments. Mr S. Frampton drew the diagrams. The Central Research Fund, University of London, financed the cutting of thin sections.

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Gene frequency changes and adaptation in marine cockles

Elizabeth Gosling

Zoology Department, University College, Galway, Ireland

When closely related species overlap geographically, a change of character state may occur in one species which is directly attributable to the presence in the same environment of an ecologically similar species^{1,2}. Most reports of this phenomenon—called character displacement—have been concerned with divergence, rather than convergence, in morphological characters between species in the zone of sympatry. There has been only one report on genetic displacement at the allelic level³. In this, Murphy observed a divergence in allele frequency at a leucine aminopeptidase locus in sympatric species of marine limpets which he interpreted as allelic accommodation in response to interspecific competition³. I report here a study of two closely related species of cockles, *Cerastoderma edule* and *Cerastoderma glaucum*, in which *C. glaucum* exhibits a convergence in allele frequency at the phosphoglucumutase (Pgm) locus when it is found intermixed with *C. edule*.

In the British Isles, *C. edule* is widely distributed intertidally whereas *C. glaucum* is found in stagnant saline pools isolated from tidal influences^{4,5}. However, occasionally the two species are found together intertidally, apparently occupying identical ecological niches⁶. The two species are morphologically very similar and were separated using the characteristics outlined by Boyden⁷—shell shape, ligament length, shape of ribs, digestive gland coloration and internal shell pigmentation.

Pgm is coded at a single locus with three electrophoretic alleles, *Pgm*^A, *Pgm*^B and *Pgm*^C, present in *C. glaucum* and *Pgm*^C, *Pgm*^D, *Pgm*^E and *Pgm*^F present in *C. edule*. The allele *Pgm*^C is common to both species (Fig. 1). Because of the different electrophoretic mobilities of Pgm allozymes in *C. glaucum* and *C. edule*, the Pgm system can therefore be used in conjunction with morphological characteristics to separate the two species. In this study, unspecific samples of *C. edule* were collected from the intertidal region of the shore whereas all unspecific samples of *C. glaucum* were taken from stagnant saline pools where the cockles were permanently submerged. Table 1 presents allele frequency data in two unspecific populations of *C. edule* and *C. glaucum*. To date, all unspecific populations of either species from Irish and British sites exhibit little geographical variation in allele frequency at this locus (unpublished results). In addition to the above samples, mixed

Table 1 Pgm allele frequencies in allopatric populations of *C. edule* and *C. glaucum* from W. Ireland

	N	Allele frequency						χ^2	NS
		A	B	C	D	E	F		
<i>C. edule</i> Shannon Estuary	80	—	—	0.09	0.34	0.54	0.03	2.43	NS
<i>C. glaucum</i> Carna, Galway	95	0.0	0.68	0.32	—	—	—	2.81	NS

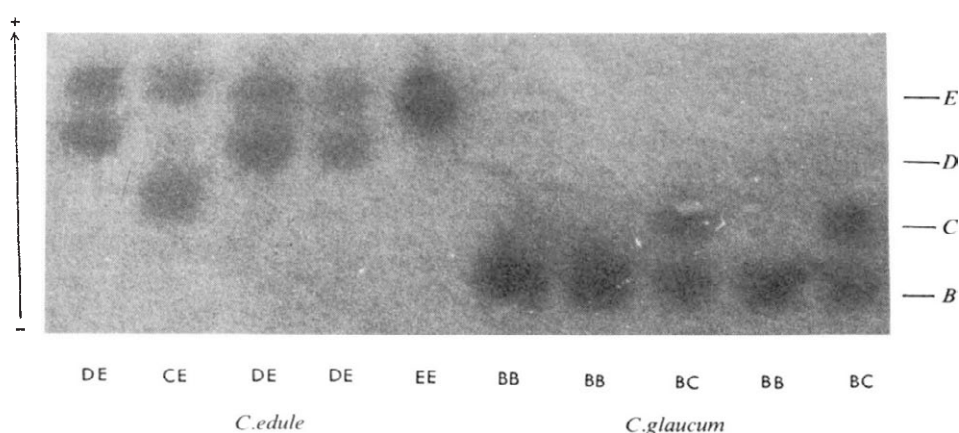
N, no. of individuals analysed. χ^2 , Goodness-of-fit of observed to expected frequencies from Hardy-Weinberg equilibrium. NS, not significant, $P > 0.05$.

populations of the two species were collected intertidally from two Irish sites (Keeraunnagark and Shannon Estuary) and two British sites (the Fleet, Dorset, and Hullbridge Ferry, Essex). At these sites, *C. glaucum* experiences quite different environmental conditions from the lagoon habitat, such as exposure to air, exposure to wave action and tidal amplitude. In the case of each mixed population, an attempt was made to collect unspecific populations of *C. glaucum* and *C. edule* from sites situated close to the mixed population. The results of the Pgm allozyme analyses of these samples are shown in Fig. 2. In some cases (see Fig. 2 c, d), it was not possible to collect unspecific samples from sites which were geographically close to a mixed population. However, the result was the same for each *Cerastoderma* assemblage, that is, allele frequency in *C. glaucum* was consistently nearer to that of *C. edule* in mixed populations of the two species by a statistically significant ($P < 0.05$) amount. Allele frequency in *C. edule* from unspecific populations, or from populations where it was mixed with *C. glaucum*, showed no significant ($P > 0.05$) difference.

Cross-fertilization can be ruled out as an explanation for the observed convergence in allele frequency. First, no Pgm enzyme genotypes indicative of hybridization were observed. Second, hybridization between these species is, in any case, unlikely. Although individuals with apparently intermediate morphological characteristics occur in mixed populations and cross-fertilization can take place in the laboratory^{7,8}, attempts⁸ to rear hybrid larvae have met with little success. In general, attempts have been less successful between species originating from mixed populations than between species taken from different geographical locations⁸. In addition, displacement in spawning times occurs in mixed populations⁹, thereby ensuring effective reproductive isolation between the two species.

The results of the present study suggest that the genetic displacement, that is, the change in allele frequency which occurs only when *C. glaucum* coexists with *C. edule*, is a genetic consequence of adaptation to the intertidal habitat. Previous reports^{3,10-12} have implicated interspecific competition as the cause of character displacement in the zone of sympatry. There is no evidence in the literature to suggest that the two cockle species compete in the zone of contact. If interspecific competition is occurring, one might expect the observed difference in

Fig. 1 Pgm alleles in *C. edule* and *C. glaucum*. Mixed samples of *C. edule* and *C. glaucum* together with unspecific samples of *C. edule* were collected from the intertidal region of the shore; unspecific samples of *C. glaucum* were collected from non-tidal lagoon sites. A portion of the foot was excised from living individuals and homogenized manually in approximately two volumes of 50 mM Tris-HCl buffer, pH 7.8 (1:1w/v). Electrophoresis was carried out at 2 °C in horizontal starch gel (Connaught) using the Tris/EDTA/maleic buffer system, pH 7.4, of Spencer *et al.*¹³. Gels were run for 14–16 h at 6.5 V cm⁻¹. Sliced gels were stained for Pgm using the paper overlay method described by Scopes¹⁴.



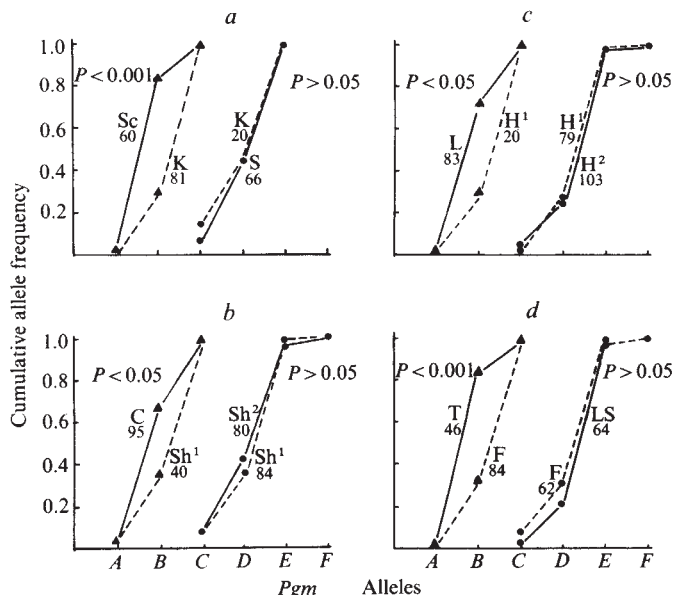


Fig. 2 Cumulative frequency of *Pgm* alleles in allopatric (—) and sympatric (---) populations of *C. glaucum* (▲) and *C. edule* (●). Samples were collected from four habitats that supported mixed populations of *C. glaucum* and *C. edule* and in each case a unispecific populations of *C. glaucum* and *C. edule* was collected from a site situated as close as possible to the mixed population. Results for each *Cerastoderma* assemblage are presented separately in a, b, c and d. *P* was derived from a two-tailed Kolmogorov-Smirnov two-sample test of the H_0 that the distribution of alleles in allopatric and sympatric populations of *C. edule* and *C. glaucum* are identical. a, S: Silverstrand, Galway; K: Keeraunnagark, Galway; Sc: Screebe, Galway. b, C: Carna, Galway; Sh¹, Sh²: Shannon Estuary, Limerick. c, L: Lady's Island, Wexford; H¹, H²: Hullbridge Ferry, Essex, UK. d, T: Tacumshin, Wexford; F: The Fleet, Dorset, England; LS: Leigh-on-Sea, Essex. Numbers beneath each site abbreviation indicate size of sample.

allele frequency to vary depending on the proportions of *C. glaucum* and *C. edule* present in mixed samples. This was not the case, as is evident from Fig. 2a and c, where the ratio of *C. glaucum* to *C. edule* in the two samples was 4:1 and 1:4 respectively. In the sample from Fleet, Dorset, individuals of *C. glaucum* were not actually intermixed with *C. edule* but were separated from them by a geographical distance of about 0.5 km. However, *C. glaucum* shows the characteristic shift in allele frequency which indicates that sympatry of the two species is not necessary to produce this effect.

We conclude that, although character divergence or convergence in sympatry can occur in response to interspecific competition there must also be occasions, for example in this study, when it occurs for reasons not directly connected with the presence of another species.

I thank N. P. Wilkins and J. McKay for useful discussions and the following people who helped in the collection of samples: D. McGrath, B. Healy, G. O'Sullivan, A. Beaumont, G. Pickett, B. Dawson and D. Seaward. This work was supported by a grant from the National Board of Science and Technology, Ireland.

Received 11 February; accepted 3 June 1980.

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Juvenile hormone mimics the photoperiodic apterization of the alate gynopara of aphid, *Aphis fabae*

Jim Hardie

Agricultural Research Council Insect Physiology Group,
Department of Zoology and Applied Entomology,
Imperial College, London SW7 2AZ, UK

Extensive investigations of the environmental factors influencing the alate-apterous dimorphism of the summer, parthenogenetic, virginoparous generations of aphids have shown that crowding has a major influence on the development of the alate morph, although temperature and nutrition are also important^{1,2}. These factors may act prenatally, postnatally or both, depending on the species. However, the physiological control of this dimorphism within the individual has not been elucidated. As the apterous adult and the larvae share certain morphological features³ it has been suggested that the hormone controlling larval-adult metamorphosis, juvenile hormone (JH), also influences development of apteriform characteristics^{4,5}. Indirect evidence from measurements of corpus allatum (CA) volumes and CA nuclei were believed to support this theory⁶ and early experiments with topically applied JH extracts and analogues seemed to divert alate development towards the apterous condition^{2,5}. Attempts to obtain more direct evidence have been negative or inconclusive⁶. Recently, Lees⁷ has questioned this hypothesis, citing the following evidence: (1) examination of more extreme forms produced by topical application of JH mimics to middle instar alatae shows them to be developing towards a fifth instar, supernumerary larva (that is juvenilization) rather than towards an apterous morph (that is apterization); (2) in an autumn, short-day-induced alate morph of *Aphis fabae*, namely the gynopara (see below), authentic apterization can be effected by postnatal exposure to long photoperiods, the first larval instar being most sensitive to this treatment. By contrast, it was the late second and the third instars that were most sensitive to juvenilization following topical application of JH analogues. It is shown here that larger doses of a more active hormone (JH1) will closely mimic the photoperiodic apterization of these gynoparae but that apterization is not due to the neotenic action of JH. Evidence is also presented suggesting that the alate-apterous dimorphism in summer, long-day generations is not controlled by JH.

In many host-alternating aphid species a separate alate, viviparous morph is produced in response to short days and gives birth to the sexual females (the oviparae). In *Aphis fabae* these gynoparae (Fig. 1a) are morphologically very similar to the alate virginoparae but may differ in the number of secondary rhinaria (placoid sensilla) on the antennae⁸. The major differences between the two alates lie in their behaviour⁸ and in the morph of their progeny.

In the experiments described below, *A. fabae* [from a clone originally isolated in 1946 (ref. 9)] were reared on germinating tick beans (*Vicia faba*) at 15 °C. Long day (LD) conditions were 16 h light: 8 h dark and short day (SD) conditions 12 h light: 12 h dark. When progeny of an apterous virginopara from an LD stock culture are reared in isolation in SD conditions they develop as apterous adults and their progeny, that is the second generation in SD, are mainly alate gynoparae but include a few late-born males and apterous virginoparae. If only the progeny born before the appearance of the males are reared at a density of between 1 and 10 per germinating bean in SD conditions then 99.8% (1,140 in 1,142) develop as gynoparae. Initially the germinating beans are 1–1.5 cm tall.

As mentioned earlier, the development of the adult alate gynopara of *A. fabae* also depends on the postnatal environ-

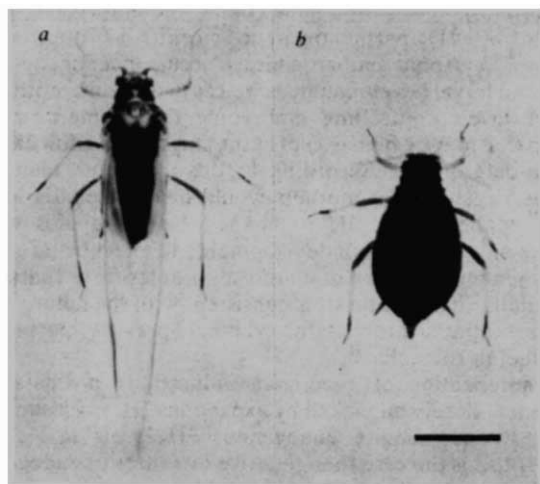


Fig. 1 *a*, The alate gynopara of *Aphis fabae* which produces sexual females (oviparae). *b*, A presumptive gynopara which has been completely apterized by topical application of 0.1 µg JH1 during the first instar. This aphid was reared in short-day conditions; similar apterae are produced when presumptive gynoparae are reared from the first instar in long-day conditions. As far as is known they are morphologically identical to apterous virginoparae. Scale bar, 1 mm.

ment⁷. If presumptive gynoparae complete larval development in LD conditions after transfer from SD during the first or early second instar a number develop into adult apterae or alate/apterous intermediates, that is apterization occurs. In extreme cases this is complete and the adult morph is identical to the apterous virginopara (see Fig. 1*b*). The proportion of adults showing various degrees of apterization is maximal if transfer to LD is effected during the first 24 h of postnatal development and decreases with the age of transfer (Fig. 2).

Lees⁷ has previously shown that by examination of fifth instar (normally adult) individuals it is possible to distinguish the more extreme forms of juvenilized alate gynoparae induced by treatment with JH mimics juvenilization and presumptive gynoparae which have been diverted along an apterous developmental pathway by postnatal exposure to LD (apterization). Less extreme apterized and juvenilized alate fifth instar individuals—those designated as 25% morphological effect—are difficult to distinguish. This category is, however, rare in the LD, 'authentic' apterization (12 of 1,017 insects transferred to LD between days 1 and 4 of postnatal development) and so when observed in the following JH experiments it was assumed to be an indication of juvenilization rather than apterization.

It was found that photoperiodic apterization could in fact be mimicked by JH (Figs 1*b*, 2). JH1 (0.1 µg, Calbiochem) in 0.1 µl acetone was applied topically to the abdomen of presumptive gynoparae, postnatal development continuing in SD conditions. The numbers of insects showing apterous characters was less than that obtained by transfer to LD conditions at the same stage of development except on the fourth day

after birth. However, the response profile was similar in both apterizing treatments. Presumptive gynopara controls either, untreated or treated with 0.1 µl of acetone, and reared in SD at <10 per bean were almost all alate adults (0.2% of individuals showed apterous characters).

JH-treated insects that were not apterized often showed juvenilization are the second and third instars, an observation for the degree of juvenility the total effect was calculated after Lees⁷ (Fig. 3). The developmental stages most sensitive to juvenilizations are the second and their instars, an observation also reported by Lees⁷. Thus the apterizing effects and the juvenilizing effects of JH treatment show different sensitivity profiles, a finding which indicates that separate target sites exist and that apterization is not due to the neotenic action of JH.

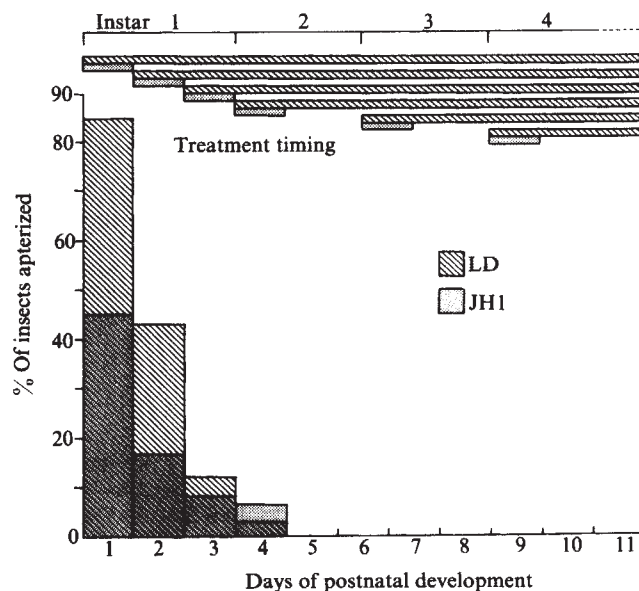


Fig. 2. The percentage of presumptive gynoparae which showed some degree of apterization when either transferred to LD conditions (hatched) or treated with a single application of 0.1 µg JH1 (stippled) at different stages during postnatal development. The JH-treated insects were reared in SD conditions throughout development. All insects were reared with an initial population of 10 per germinating bean but due to the increased mortality in JH-treated individuals there was often less than 10 for much of development. Number of insects tested in the LD treatment were: day 1, 193; day 2, 148; day 3, 143; day 4, 94; first day of the third instar, 61; first day of the fourth instar, 102. Only the survivors of the JH treatment could be scored and survival depended on the age when treated. Survivors per number treated were: day 1, 67/210; day 2, 24/454; day 3, 37/389; day 4, 77/103; first day of the third instar, 65/70; first day of the fourth instar, 102/104. Of controls dosed on day 1 with 0.1 µl acetone and reared in SD 27/30 survived; all survivors were alate gynoparae. Of untreated controls reared in SD 99.8% (971/973) were alate when reared at a density of between 2 and 10 individuals per germinating bean while 100% (142/142) were alate when reared at one per bean. These alatae are induced by the pre- and postnatal SD treatment, not by crowding.

Table 1 The apterizing effects of 0.1 µg JH1 on presumptive gynoparae less than 24 h after birth, compared with the alating effect of crowding. Development continued in SD conditions

Crowding intensity	Control survival	Experimental survival	%Insects showing apterization Controls	JH-treated
<i>a</i> Relatively uncrowded (10 aphids per germinating bean)	(404/410) = 98.5%	(67/210) = 31.9%*	0.5%	44.8%
<i>b</i> Crowded (200 aphids per bean stipule)	(166/250) = 66.4%	(13/250) = 5.2%	0%	0%

The crowding intensity was increased in *b* compared with *a* not only by increasing the number of aphids in the colony but also by using a bean stipule (growing tip removed). These plants showed retarded growth during the experiments when compared with the germinating beans. The increase in density of crowding leads to a decreased survival in both control and experimental animals. Control animals are untreated.

* These values are the same as those of day 1, JH-treated insects in Fig. 2.

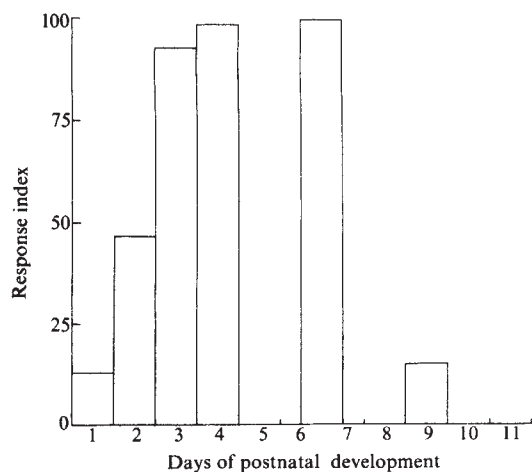


Fig. 3 The total degree of juvenilization exhibited by aphids that were not apterized by JH treatment in Fig. 2. The response index was calculated after Lees⁷, each insect being assigned to one of five groups of 'morphological effect' namely 0, 25, 50, 75 and 100%. 0% was a normal alate while 100% was a fifth instar supernumerary nymph, the other groups falling between these extremes. The response index was calculated as $\Sigma(\% ME \times n)/N$; ME, morphological effect; n , number showing this grade of response; and N , total number of individuals. Due to the different mortalities and apterization caused by the treatments the N varied as follows: day 1, 37; day 2, 20; day 3, 34; day 4, 72; first day of the third instar, 65; first day of the fourth instar, 102.

In LD, virginoparous aphid generations wing production is induced by crowded conditions, postnatal crowding being a predominant factor in *A. fabae*¹⁰. It was therefore of interest to test whether the LD apterization of presumptive gynoparae observed in relatively uncrowded conditions (10 per germinating bean) could be opposed by the alating effect of crowding. In one experiment where day 1 presumptive gynoparae were crowded on to a single bean stipule (growing tip removed) and transferred to LD, 100% (126 of 126) of the adult survivors were alatae, in a second experiment 88% (83 of 94) were alatae. In uncrowded conditions only 15% (28 of 193) were alatae (Fig. 2). Thus the apterization induced by LD could be cancelled out by crowding.

Experiments were also designed to test the effectiveness of apterization by exogenous JH against the alating effect of crowding. Fifty first-day presumptive gynoparae were treated with 0.1 µg of JH1 and placed on a bean stipule with 50 first-day control aphids marked by the removal of the left antenna. After leaving these insects to settle overnight a further 100 first-day larvae with the right antenna removed were added to the same stipule to act as an additional crowding agent. This crowded colony was allowed to develop in SD conditions and scored for alate/apterousness in the fourth or fifth instar. Due to the large residual effects of the JH1 during the larval instars most sensitive to juvenilization, the fifth instar individuals, in these experiments, were all supernumerary nymphs. However, alate individuals could easily be distinguished from apterized insects. Table 1 summarizes the results of five such experiments (b) in comparison with a similar uncrowded series (a). It can be seen that the increase in intensity of crowding decreases the survival of both control and JH-treated individuals but all survivors in the crowded JH-treated experiments were alatae while 45% were apterized in uncrowded experiments. Control experiments showed there to be no differential mortality between alate and apterous morphs. Thus the apterization of alate gynoparae induced by either LD or JH treatment may be cancelled out by crowding.

Recent examination of the role of JH in controlling aphid polymorphism, as investigated by topical application of JH or analogues, indicates that in addition to influencing larval-adult

metamorphosis it has a possible role in the embryonic determination of LD parthenogenetic morphs as opposed to oviparae^{11,12}. Aphid embryogenesis occurs paedogenetically throughout larval development as well as in the adult mother, so that JH levels controlling embryonic development in LD virginoparae may be high except at the stage when adult characters are determined. According to this view both alate and apterous virginoparous mothers would have raised JH levels. Topical application of JH to larvae of these aphids would influence only larval-adult development, no effect being expected on the determination of alateform or apteriform characters of the adult. This hypothesis is consistent with the failure of JH to induce aptera production, whether pre- or postnatally controlled, in LD aphids^{6,12}.

The apterization of gynoparae induced by postnatal LD treatment is closely mimicked by exogenous JH, suggesting that LD conditions increase endogenous JH levels in *A. fabae* larvae. If this is the case then putative low titres of endogenous JH induced by SD exposure would promote both wing development and the embryonic development of oviparae. Both LD- and JH-induced apterization involves a switch in embryonic development from oviparae to parthenogenetic viviparae (ref. 7 and unpublished results). The observation that crowding can cancel out the apterization induced by either LD or JH points to the existence of a separate factor which controls the alate-apterous dimorphism in LD, virginoparous aphids.

I thank Professors A. D. Lees and B. Johnson for advice and Professor J. S. Kennedy for comments on the manuscript.

Received 14 November 1979; accepted 2 June 1980.

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Two distinct patterns of visual evoked response asymmetry in human albinism

W. M. Carroll, B. S. Jay, W. I. McDonald & A. M. Halliday

The National Hospital for Nervous Diseases, Queen Square, London WC1 3BG and Moorfields Eye Hospital, City Road, London EC1, UK

Results are presented which confirm that in human albinos each cerebral hemisphere receives a predominantly monocular input from the contralateral eye, giving rise to an asymmetry of the visual evoked potential (VEP) to whole-field stimulation which is similar to that for stimulation of the temporal half-field in the same individual. The further finding of two distinct, and in some respects opposite, types of topographical asymmetry raises the possibility that there are two anatomically distinct variants in the retinocortical projection in human albinism, as there are in the Siamese cat.

Albinism is associated with a peculiar anatomical anomaly of the visual pathways resulting from a misrouting of the retinogeniculate projections. In the extensively studied Siamese cat, not only is the whole of the temporal half-field of each eye projected to the opposite hemisphere, but also up to 20° of the nasal half-field. Each hemisphere therefore receives a predominantly monocular input from the contralateral eye, with only the more peripheral parts of the nasal field represented

ipsilaterally¹. A similar anomaly appears to be associated with albinism in all the mammalian species so far studied, including albino rats, guinea pigs, rabbits, and mink²⁻⁷. There is now also evidence for a similar anomaly in man⁸.

The misrouting of the retinocortical projection results in a partial disorganization of the normal cortical retinotopic representation in the contralateral occipital lobes, and it appears from anatomical and electrophysiological studies in Siamese cats that the visual system deals with the abnormal projection from the nasal field of the contralateral eye in two distinct ways. In one, the 'Boston' pattern, the anomalous central 20° of the nasal field has a separate representation in V1 and V2 (areas 17 and 18) interpolated on either side of the 17/18 border, so that the representation of the fixation point or the area centralis is shifted away from the common border of areas 17 and 18 by the abnormal input⁹. In the other variant, the 'mid-Western' pattern, the area centralis is still positioned, as in the normal cat, at the 17/18 border, and the abnormal input from the contralateral temporal hemiretina overlies it, so that mirror-symmetrical points to either side of the fixation point go to almost identical positions on the cortex, rather as the homologous points from homonymous half-fields in each eye converge in the normal cat¹⁰. A further feature of the mid-Western pattern is the 'suppression' or reduced number of cortical units in the area of cortex receiving the abnormal input. There has been some evidence suggesting that in some cases both types of anomaly may co-exist in the same individual¹¹.

Creel *et al.* have recorded the visual evoked potential in human albinos and report that up to 70% of individuals have an abnormal response with evidence of missing components from the hemisphere receiving the uncrossed visual pathway¹²⁻¹⁴. Since pattern reversal VEPs have proved particularly consistent in normal healthy subjects, and the responses from each half-field show a characteristic asymmetry in all normal individuals^{15,16}, we have recorded the VEPs in 15 human albinos to reversal of a black-and-white checkerboard pattern of 50'

checks, using a multi-channel montage to examine in detail the topographical distribution of the response over the back of the head. Three had X-linked, and five had autosomal recessive ocular albinism and seven were oculocutaneous albinos (tyrosinase positive in six). The visual acuities were 6/36 or worse bilaterally in nine subjects and the remainder had best monocular acuities of between 6/9 and 6/24. No differences were found in the size of the central nasal and temporal visual fields and all but three had varying degrees of pendular or jerk nystagmus when the eyes were in the primary position. In all, the nystagmus diminished considerably during VEP recording. Recordings where fixation (monitored throughout) was judged inadequate or nystagmus excessive or which were not reproducible were rejected.

All the monocular responses to a circular stimulus of 16 degree radius showed a marked asymmetry which clearly distinguished them from those of normal subjects (Fig. 1). This whole-field response asymmetry invariably resembled the asymmetry produced by stimulation of the temporal half-field in the same eye. However, two quite distinct, and in some respects opposite, types of asymmetry were encountered in the albino group. In five subjects the P100 component (major positivity) was ipsilateral to the stimulated eye or temporal half-field, being similar in distribution to the P100 of the temporal half-field response of the normally pigmented subject. In nine others the distribution of the P100 was the reverse of that found in normal subjects with this component appearing in the channels contralateral to the stimulated eye. One individual showed a mixture of the two patterns, the P100 being ipsilateral for the left eye responses and contralateral for the right eye responses. In both groups of subjects the P100 to nasal half-field stimulation showed an almost identical distribution to that for the temporal half-field and whole-field responses from the same eye. In general, the amplitude of the nasal field P100 was smaller than the temporal field P100, this difference being most marked in the first group of five subjects. There was, however, no

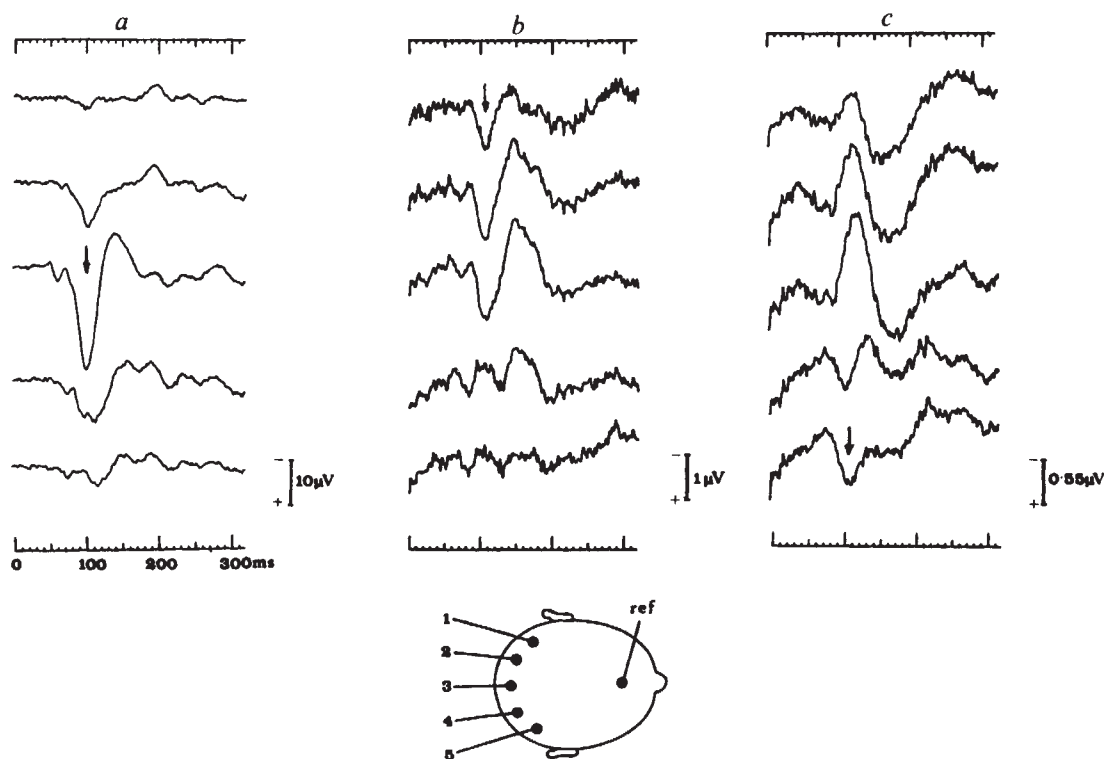


Fig. 1 Left eye whole-field (16 deg radius) VEPs from: *a*, a normal subject; *b*, the grand average of the 5 albino subjects with a P100 (arrow) distribution ipsilateral to the stimulated left eye and *c*, the grand average of the 9 albino subjects with a contralateral P100 (arrow) distribution. Note in the normal subject the larger amplitude and symmetrical distribution of the P100 about the midline. The midline electrode of the transverse chain of 5 is situated 5 cm above the inion.

obvious association between the various types of albinism and the two patterns of VEP asymmetry. The mean amplitude of the VEPs to all fields of stimulation in the albinos was significantly smaller than in the normal population, in line with the lowered visual acuity characteristic of this group.

Received 21 April; accepted 18 June 1980.

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Ethyl β -carboline-3-carboxylate shows differential benzodiazepine receptor interaction

Mogens Nielsen & Claus Bråstrup*

Psychopharmacological Research Laboratory, St Hans Mental Hospital, Department E, DK-4000 Roskilde, Denmark

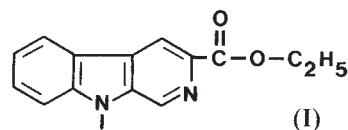
* Research Laboratories, A/S Ferrosan, Sydmarken 1-5, DK 2860 Soeborg, Denmark

High-affinity binding of ^3H -diazepam and ^3H -flunitrazepam has provided evidence for the presence of benzodiazepine receptors on brain neurones^{1–6}. Pharmacological evidence showing a clear correlation between receptor affinity and *in vivo* pharmacological potency for several benzodiazepines⁷ and a link between benzodiazepine receptors and GABA (γ -aminobutyric acid) receptors at the molecular level^{8,9}, indicates that these receptors are relevant to the pharmacological and clinical effects of benzodiazepines. In searching for possible endogenous ligands for benzodiazepine receptors we have recently isolated ethyl β -carboline-3-carboxylate (β -CCE) from human urine and brain, and shown that β -CCE has a higher affinity than diazepam for brain benzodiazepine receptors¹⁰. β -CCE itself is probably not present in the brain, but may be closely related to an endogenous benzodiazepine receptor ligand¹⁰. We report here that β -CCE, in contrast to benzodiazepines, can distinguish clearly between benzodiazepine receptors in cerebellum and hippocampus. This result strongly indicates that benzodiazepine receptors are not a single class of non-interacting entities. It has not been possible to determine whether two distinct receptors are present and/or whether true negative cooperativity exists among hippocampal, but not cerebellar, benzodiazepine receptors.

Purification of human urine, including treatment with hot ethanol, yielded 1.78 mg β -CCE¹⁰, which was stored in 50% ethanol at 0–4 °C for more than 2 months without loss of activity. Its identity was confirmed by *de novo* synthesis and crucial experiments were repeated with synthetic material (A/S Ferrosan). Crude membrane fractions from different brain regions were prepared by homogenization by an Ultra Turrax homogenizer in 10 vol of ice-cold 25 mM potassium phosphate buffer, pH 7.1, followed by dilution to 100 v/w original tissue. Aliquots of 500 μl were used directly in binding experiments. β -CCE test solution (25 μl) was added to assays just before ^3H -flunitrazepam (25 μl , 87 Ci mmol⁻¹, NEN) and samples

were incubated at 0 °C for 40 min. Specific ^3H -flunitrazepam binding was estimated by conventional filtration techniques² using 3×10^{-6} M diazepam to assess nonspecific binding.

The first indication that β -CCE did not interact like a simple competitive inhibitor on a single class of benzodiazepine receptor came from Scatchard analysis of ^3H -flunitrazepam binding to rat forebrain. These plots were curvilinear in the presence of β -CCE¹⁰ whereas plots without β -CCE never (>200 experiments) exhibited curvatures. A regional study on rat brain revealed that the curvature was most pronounced in the hippocampus and that no curvature was detected in cerebellum (Fig. 1). Other brain regions, such as frontal cortex, occipital cortex, striatum, hypothalamus and thalamus all exhibited curvilinear Scatchard plots for ^3H -flunitrazepam in the presence of β -CCE (data not shown).



(I) Ethyl β -carboline-3-carboxylate

(3-carboethoxy-9H-pyrido [3,4-b] indole; β -CCE)

Fig. 1 Ethyl β -carboline-3-carboxylate (3-carboethoxy-9H-pyrido[3,4-b]indole; β -CCE).

Benzodiazepine receptors can exist in several conformations which exhibit different affinities. Various proportions of alternative conformational states in different brain areas might account for the observed difference between cerebellum and hippocampus. Mixed type inhibition by β -CCE, however, was consistently seen in hippocampal synaptosomal membranes which had been prepared in various ways known to favour different conformational states—for example, membranes prepared in sucrose²; in membranes washed five times (Tris-citrate)¹¹; in washed membranes which were 'activated' by 3×10^{-5} M GABA^{8,11,12}; in membranes treated with Triton X-100 (0.1%)¹³; in partially heat-inactivated membranes (Tris-HCl; 15 min at 60 °C)¹⁴; in crude membranes prepared in a physiological buffer (122 mM NaCl, 4.8 mM KCl, 0.97 mM CaCl₂, 1.2 mM MgSO₄, 16 mM phosphate, 1 mM ascorbic acid, 11 mM glucose) and in crude membranes activated by 30 mM potassium iodide¹⁵ (data not shown). These data indicate that curvilinear Scatchard plots were not dependent on any previously described conformational heterogeneity.

Besides the induction by β -CCE of curvilinear Scatchard analysis, hippocampal benzodiazepine receptor binding differed from cerebellar binding in the displacement potency of β -CCE for ^3H -flunitrazepam. The IC₅₀ value for β -CCE, which is the concentration displacing 50% of specific ^3H -flunitrazepam binding, was only 0.36 ng ml⁻¹ (range 0.22–0.57 ng ml⁻¹, Hill coefficient 0.95 ± 0.02 , mean $\pm$ s.e.m., $n = 4$) in cerebellum compared with 2.7 ng ml⁻¹ (range 1.7–3.6 ng ml⁻¹, Hill coefficient 0.75 ± 0.04 , mean $\pm$ s.e.m., $n = 4$) in hippocampus (Fig. 2). β -CCE was more potent in displacing ^3H -flunitrazepam in cerebellum than in hippocampus irrespective of the membrane preparation used (see above, data not shown). Furthermore, cerebellar and hippocampal tissue from mouse and pig exhibited about the same difference in affinity for β -CCE as did rat brain tissue (data not shown).

The almost sevenfold higher affinity of β -CCE for cerebellar than for hippocampal ^3H -flunitrazepam binding does not unequivocally show that β -CCE possesses higher affinity for cerebellar receptors because IC₅₀ values depend on the K_D value for the ^3H -ligand. The K_D value for ^3H -flunitrazepam binding was, however, quite similar in cerebellum ($K_D = 1.40 \pm 0.08$ nM; mean $\pm$ s.e.m., $n = 10$) and hippocampus ($K_D = 1.34 \pm 0.04$ nM; mean $\pm$ s.e.m., $n = 16$), excluding regional differences in the affinity for ^3H -flunitrazepam as a major contributor to the observed regional differences.

The present results clearly show that benzodiazepine receptors in cerebellum and hippocampus are not identical. One interpretation would be that one class of receptor is most abundant or exclusively present in cerebellum and that this class exhibits high affinity for β -CCE. Two or more classes of receptors are present in hippocampus; at least one of them exhibits reduced affinity for β -CCE. The observation of Hill coefficients below unity is compatible with the presence of more than one receptor species in the hippocampus with different affinities. The possible occurrence of receptor heterogeneity in the central nervous system was proposed, based on experimental evidence showing biphasic dissociation curves¹⁴, polyphasic heat inactivation curves¹⁴ and flat ^3H -diazepam displacement curves with Hill coefficient below unity for some new triazolopyridazines¹⁴. Furthermore, curvilinear Scatchard plots for ^3H -diazepam were found in the brain of lower vertebrates¹⁶. Direct evidence for benzodiazepine receptor heterogeneity in hippocampus has been obtained in photoaffinity labelling experiments using ^3H -flunitrazepam. SDS-electrophoresis of these irreversibly radiolabelled receptors showed two molecular species with different molecular weights (MWs) (W. Sieghart and M. Karobath, personal communication).

An alternative explanation for the present results that is compatible with both the curvilinear Scatchard analysis and the Hill coefficients below unity would be that benzodiazepine receptors in hippocampus, in contrast to cerebellar receptors, exhibit true negative cooperativity. Solubilization of benzodiazepine receptors reveals MWs of either 50,000 (ref. 17), 200,000 (ref. 18) or 110,000 (C.B. *et al.*, unpublished), depending on the procedures applied, indicating the possible presence of dimers or tetramers. Receptors composed of polymer subunits in hippocampus might possess mutually interacting sites, so that binding of β -CCE (but not of a benzodiazepine) to one site decreases the affinity of the adjacent receptor site for benzodiazepines. Apparent negative cooperativity has previously been observed for the interaction of a neurotransmitter agonist with receptors identified by labelled antagonists^{19,20}. It is tempting to speculate that β -CCE is an 'agonist' to 'antagonistic

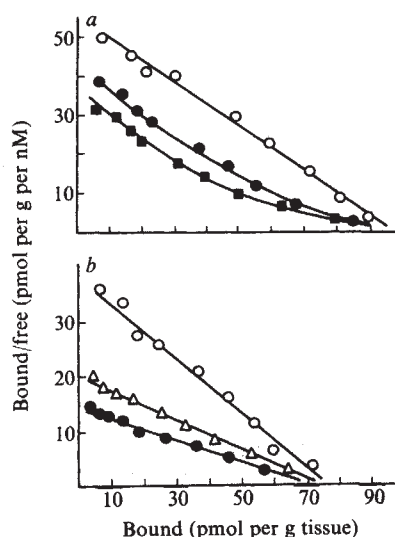


Fig. 2 *a*, Scatchard analysis of specific ^3H -flunitrazepam binding in rat hippocampus. Crude membrane fractions (2 mg original tissue per assay) were incubated with ^3H -flunitrazepam (0.2–20 nM) in duplicate as described in the text. Blanks contained 3×10^{-6} M diazepam. Control (no addition of inhibitor), $\circ$; addition of ethyl β -carboline-3-carboxylate, $\bullet$ (0.56 ng ml $^{-1}$) and $\blacksquare$ (1.1 ng ml $^{-1}$). The results are from a single experiment which was repeated three times with very similar results. *b*, Scatchard plot of ^3H -flunitrazepam binding in rat cerebellum estimated as described in *a*. Control (no addition of inhibitor) $\circ$; addition of ethyl β -carboline-3-carboxylate $\triangle$ (0.28 ng ml $^{-1}$) and $\bullet$ (0.56 ng ml $^{-1}$). The experiment was repeated three times with similar results.

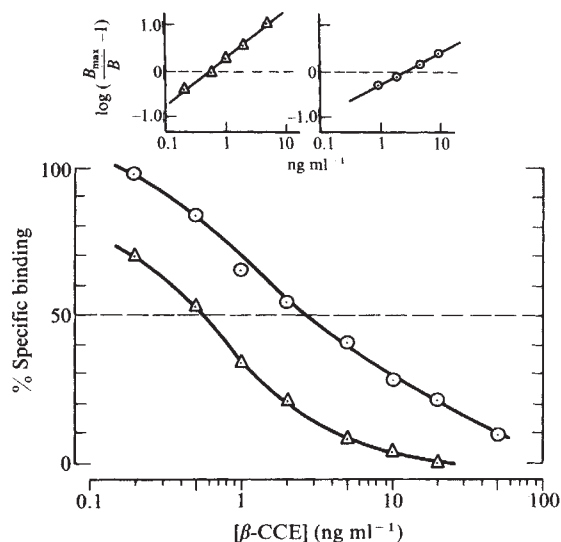


Fig. 3 Inhibition of ^3H -flunitrazepam binding (1 nM) to crude membrane fractions of rat hippocampus ($\circ$) and cerebellum ($\triangle$) by increasing concentrations of ethyl β -carboline-3-carboxylate (0.2–50 ng ml $^{-1}$) added in duplicate. Total binding in hippocampus and cerebellum was 16,200 and 11,600 c.p.m. per assay, respectively. Nonspecific binding was 6–11% of total binding. Plots are representative of four separate experiments. Inset: Hill plots of the above data. Hill coefficients were 0.95 ± 0.02 and 0.75 ± 0.04 (mean $\pm$ s.e.m. of 4 values) for cerebellum and hippocampus, respectively.

sites' labelled by benzodiazepines. Studies of several drug receptors show that GTP changes the affinity of agonist binding to antagonist sites^{21,22}. We found no effects of GTP (10^{-5} – 10^{-4} M) on the affinity of β -CCE in hippocampus or cerebellum (data not shown), indicating that any cooperative interaction is not dependent on GTP-dependent coupling of the benzodiazepine receptor to adenylate cyclase.

Much work has gone into developing benzodiazepine derivatives with more selective pharmacological actions. Clinically available benzodiazepines apparently always exhibit anxiolytic, hypnotic and anticonvulsant properties in accordance with their lack of selectivity for any particular class of benzodiazepine receptors. New classes of drugs, such as triazolopyridazines²³ and derivatives of β -carboline-3-carboxylic acid, may interact preferentially with particular classes of receptor and exhibit new profiles of action.

Received 17 March; accepted 2 June 1980.

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Adrenal corticoids regulate sensitivity of noradrenaline receptor-coupled adenylate cyclase in brain

Philip L. Mobley & Fridolin Sulser

Psychopharmacology Research Center, Tennessee Neuropsychiatric Institute, 1501 Murfreesboro Road, Nashville, Tennessee 37217

Considerable evidence indicates that alterations in the availability of noradrenaline (NA) at postsynaptic sites can cause compensatory changes in the NA stimulated cyclic AMP generating system in various brain regions. Thus, an increased responsiveness to NA has been reported in the limbic forebrain and cortex following reserpine or 6-hydroxydopamine¹⁻³. Conversely, manipulations which increase the availability of NA at receptor sites (monoamine oxidase (MAO) inhibitors, electroconvulsive treatment (ECT), tricyclic antidepressants) cause a decrease in responsiveness⁴⁻⁶. This up and down-regulation of central noradrenergic sensitivity is generally linked to changes of the density of β -adrenergic receptors⁷⁻¹⁰. The findings that psychotropic drugs which can either precipitate (reserpine) or alleviate (tricyclic antidepressants, MAO inhibitors and also ECT) depressive states cause opposite changes in the NA receptor-coupled adenylate cyclase system in brain have provided the basis for a revised catecholamine hypothesis of affective disorders¹¹. It has also been suggested that the pituitary adrenal axis may have a role in mood disorders¹². Since manipulation of the levels of corticosterone through bilateral adrenalectomy can alter the sensitivity of catecholamine-sensitive adenylate cyclase systems in preparations from liver^{13,14} and adipose tissue¹⁵, we have tried to determine if corticosterone could regulate the NA receptor-coupled adenylate cyclase system in brain tissue. We now report that adrenal corticoids can alter the sensitivity of this system to NA.

Male Sprague-Dawley rats weighing 200–250 g were used for all experiments. Animals were anaesthetized with ether and the adrenal glands removed bilaterally. In sham-operated rats, the glands were located but not removed. All animals were given free access to both tap water and 3% saline as it has been demonstrated that adrenalectomized animals develop a salt taste and are thus able to regulate their own salt intake¹⁶. Animals had free access to a standard laboratory diet (Purina) and were maintained in standard laboratory conditions (12 h light-dark cycle). Two weeks following surgery, the animals were decapitated, the brains rapidly removed and the frontal cortex dissected. For studies of the NA-sensitive adenylate cyclase system, the cortex was sliced in one plane at 0.3-mm intervals and tissue slices were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4, 37°C) as previously described¹⁷. The cyclic AMP response to NA was established by exposing the tissue slices to the agonist for 10 min. Following aspiration of the buffer solution, the slices were frozen in liquid nitrogen and homogenized in 0.3 M perchloric acid. Cyclic AMP was isolated by ion exchange chromatography as previously described¹⁷ and measured by the protein binding assay of Gilman¹⁸. The effect of adrenalectomy on β -adrenergic receptors in the frontal cortex was assessed with ³H-dihydroalprenolol (DHA) based on the method of Bylund and Snyder¹⁹. Binding curves were determined over a ligand concentration range of 0.3 to 14 nM in a final volume of 1 ml. Nonspecific binding was determined in the presence of 10 μ M ($\pm$)-propranolol. The dissociation constant (K_d) and maximum binding (B_{max}) values were determined by Scatchard analysis²⁰. Protein was assayed by the method of Lowry *et al.*²¹. The significance of the data was evaluated by the 2-tailed Student *t*-test. Drugs were obtained as follows:

noradrenaline HC1, isoprenaline HC1, corticosterone, protein kinase from beef heart, cyclic AMP (Sigma) ³H-cyclic AMP and ³H-dihydroalprenolol (both NEN).

Two weeks following bilateral adrenalectomy the NA-induced cyclic AMP accumulation was significantly higher in slices from the rat frontal cortex as compared with the cyclic AMP accumulation in cortical slices from sham-operated rats. Dose-response curves comparing responses in tissue from adrenalectomized and sham-operated animals suggest that this change in sensitivity to NA is due to a shift in the maximum responsiveness of the system (Fig. 1). Significant differences in cyclic AMP accumulation occurred at NA concentrations of 100 and 1,000 μ M (68% and 44% respectively). No significant changes in the EC₅₀ values were observed. In addition, bilateral adrenalectomy appeared not to have any effects on basal levels of cyclic AMP (Table 1).

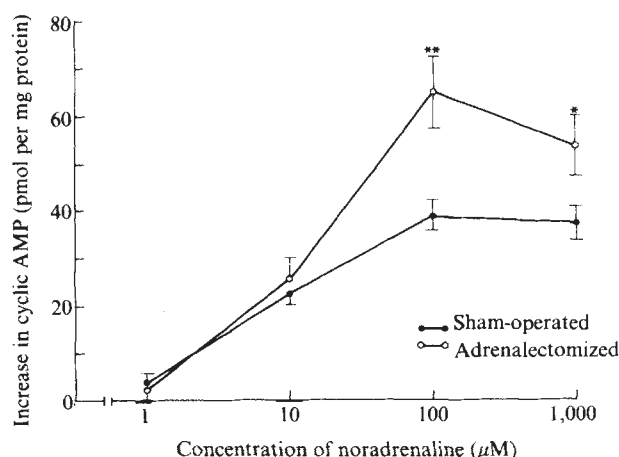


Fig. 1 Effect of adrenalectomy on the noradrenaline-induced increase in cyclic AMP accumulation in slices of rat frontal cortex. Slices of the frontal cortex from either sham-operated or adrenalectomized rats (2 weeks post-operative) were exposed to varying concentrations of noradrenaline for 10 min. The increase in cyclic AMP represents agonist-stimulated cyclic AMP levels minus basal levels (15.5 ± 1.8 and 13.1 ± 1.8 pmol cyclic AMP per mg protein in tissue from sham-operated and adrenalectomized animals respectively). Each point represents the mean of 13 to 16 samples $\pm$ s.e.m. obtained in four separate experiments. EC₅₀ values were calculated to be 7.0 ± 2.2 ($n = 4$) and 12.0 ± 2.5 ($n = 4$) μ M in tissue from sham-operated and adrenalectomized animals respectively. * $P < 0.05$, ** $P < 0.01$, as compared with sham-operated animals.

Corticosterone (10 mg per kg) administered to adrenalectomized rats for 5 days before death completely abolished the difference in sensitivity to NA (Table 1). Similar treatment elicited only a slight nonsignificant lowering of the accumulation of cyclic AMP in sham-operated rats. Again, no significant changes were observed in basal levels of cyclic AMP with any of these treatments. Also, bilateral demedullation did not produce significant changes in the sensitivity of the system to NA. The results indicate that the observed change in sensitivity to NA after bilateral adrenalectomy is a consequence of the removal of the adrenal corticosteroids.

Although the cyclic AMP accumulation elicited by NA was significantly altered following adrenalectomy, no significant changes were observed in the response to maximally effective concentrations (10 μ M) of isoprenaline: 27.6 (s.e.m., 4.2 , n , 18) and 22.5 (s.e.m., 2.6 , n , 18) pmol cyclic AMP per mg protein in slices from sham-operated and adrenalectomized animals respectively. Only slight, nonsignificant changes occurred in the B_{max} value for specific ³H-DHA binding. In tissue from sham-

Table 1 Effect of corticosterone on adrenalectomy induced change in sensitivity of the noradrenaline sensitive adenylate cyclase system in frontal cortex

	Cyclic AMP response to noradrenaline (100 μ M) (pmol per mg protein $\pm$ s.e.m.)	
	Minus corticosterone	Plus corticosterone
Sham-operated	48.8 $\pm$ 4.8	40.0 $\pm$ 3.2
Adrenalectomized	68.7 $\pm$ 4.1*	37.1 $\pm$ 1.9†

Corticosterone (10 mg per kg) or vehicle (corn oil) were administered subcutaneously to adrenalectomized and sham-operated rats for 5 days preceding death (injections started 9 days post-operation). The cyclic AMP response to 100 μ M noradrenaline equals the agonist-stimulated level minus the basal level. Basal levels in pmol per mg protein $\pm$ s.e.m. were as follows: sham-vehicle, 14.5 $\pm$ 1.1; sham-corticosterone, 16.6 $\pm$ 1.4; adrenalectomy-vehicle, 14.7 $\pm$ 1.1, adrenalectomy-corticosterone, 13.2 $\pm$ 1.0. Each value represents the mean of 8 samples for basal levels and 12 samples for agonist-stimulated levels.

* $P < 0.01$, as compared with sham-operated rats not receiving corticosterone.

† $P < 0.001$, as compared with adrenalectomized rats not receiving corticosterone.

operated rats $B_{\max}$ was 244 fmol per mg protein (s.e.m., 24; n , 6) versus 276 fmol per mg protein (s.e.m., 24; n , 6) in tissue from adrenalectomized rats. Also, no significant changes in K_d values were found: 2.59 nM (s.e.m., 0.08; n , 6) and 2.83 nM (s.e.m., 0.08; n , 6) for sham-operated and adrenalectomized animals respectively.

In the liver, it has been demonstrated that the enhanced cyclic AMP response after adrenalectomy is associated with an increase in the number of β -adrenergic receptors²². The results of the present studies suggest that the altered cyclic AMP response to NA in cortical slices following bilateral adrenalectomy is mainly a consequence of changes in a non- β -receptor-mediated portion of the adrenergic cyclic AMP generating system.

Interestingly, it has been reported that chronic stress can decrease the sensitivity of this system²³. Since chronic stress and antidepressant treatments both produce subsensitivity of the NA receptor-coupled adenylate cyclase system, Stone²⁴ has proposed subsensitivity to NA as a link between adaptation to stress and antidepressant therapy. Although it can be concluded from the present studies that alterations in the level of adrenal steroids can either directly or indirectly alter the sensitivity of the NA receptor-coupled adenylate cyclase system in brain tissue, both the molecular mechanisms accounting for the observed sensitivity changes and the role of the hypothalamus-pituitary-adrenal axis in affective disorders remain topics for further study.

This study was supported by USPHS grant MH-29228, a Fellowship by Hoffmann-La Roche (P.L.M.) and by the Tennessee Department of Mental Health and Mental Retardation.

Received 21 January; accepted 9 June 1980.

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Suppression of *in vitro* lymphocyte reactivity by cyclosporin A: existence of a population of drug-resistant cytotoxic lymphocytes

T. Horsburgh*, P. Wood & L. Brent

Department of Immunology, St Mary's Hospital Medical School, London W2 1PG, UK

Cyclosporin A (CS-A) is an unusual endecapeptide isolated from the fungi *Cylindrocarpum lucidum* Booth and *Trichoderma polysporum*¹. It is a potent immunosuppressive drug that prevents rejection of kidney²⁻⁴ and heart^{5,6} allografts whilst having a low myelotoxicity⁷. Its mode of action is still unclear but its main target appears to be the T lymphocyte⁸. The mixed lymphocyte reaction (MLR) and cell-mediated lymphocytotoxicity (CML) used here are generally regarded as *in vitro* correlates of allograft rejection⁹. Our data show that CS-A is a powerful inhibitor of MLR reactivity, regardless of whether the cells were obtained from normal or presensitized donors; that the CML of lymph node cells is likewise totally inhibited if the drug is added early during cell culture; and that, by contrast, the cytotoxic response of spleen cells from presensitized but not from normal mice is only partially inhibited, even with a tenfold increase in dose. It is therefore suggested that there exists a population of cytotoxic spleen cells that is relatively resistant to the action of this drug.

Cyclosporin A (CS-A) was added at different times after initiation of MLR between CBA (H-2^k) responder cells and irradiated BALB/c (H-2^d) or DBA/2 (H-2^d) splenic stimulator cells (Fig. 1). The presence of CS-A from the beginning of culture caused almost complete inhibition of ³H-thymidine (³H-Tdr) incorporation. Addition of the drug at later times was also effective in inhibiting further proliferative responses. (All experiments were done at least twice, with closely similar results.)

Inhibition of MLR was dose-dependent (Fig. 2). At an initial concentration of either 0.125 or 0.25 μ g ml⁻¹, CS-A severely inhibited the primary MLR response whereas lower concentrations, 0.05 and 0.01 μ g ml⁻¹ (the latter not shown), were far less effective. MLR cultures of lymph node cells from mice presensitized *in vivo* one month before, by either a skin graft or via allogeneic spleen cells injected intraperitoneally, showed a similar response to the drug. Here the untreated control cells from presensitized donors produced an earlier though lower peak in the MLR. Depression of MLR after *in vivo* presensitization has previously been described by Cooley¹⁰, who suggested that it was brought about by either a redistribution of subpopulations of lymphocytes in the animal or the elimination, during culture, of some of the stimulator cells by preformed cytotoxic cells. In our experiments the presensitized cells gave normal primary MLR responses to third-party spleen cells (C57B1, H-2^b) and these cultures were similarly inhibited by the same concentrations of CS-A (not shown).

* Present address: Department of Surgery, Leicester General Hospital, Leicester, UK.

The same range of CS-A concentrations also impaired the generation of cytotoxic lymphocytes from cultures of both normal and presensitized spleen and lymph node cells (Fig. 3). Thus, cytotoxicity after 5 days of culture was abolished when CS-A was added on day 0. Whereas addition of the drug on day 1, and to a lesser extent on day 2, partially inhibited cytotoxicity, such an effect was no longer demonstrable when the drug was added on day 3. Similar results were obtained when the cells were cultured for 6 rather than 5 days, indicating an early and lasting impairment of the capacity to produce cytotoxic cells. Addition of CS-A at later times affected the cytotoxic response of normal cells more strongly than that of presensitized cells and this probably reflects the more rapid development of cytotoxic cells in the latter¹¹. Ethanol, the CS-A solvent, did not affect CML responses at the appropriate concentration; nor was the drug directly cytotoxic as judged by the low background of radioactivity released by ⁵¹Cr-labelled target cells when incubated with the drug at $1 \mu\text{g ml}^{-1}$. However, when cytotoxic cells were assayed in the presence of $1 \mu\text{g ml}^{-1}$ CS-A, ⁵¹Cr release was reduced from a control value of 68.3% to $34 \pm 3\%$. This suggests that mature cytotoxic lymphocytes are susceptible to the action of CS-A but that they are less vulnerable than MLR and/or CML precursor cells. It is unlikely that CML suppression was due to retention of the drug, because addition of CS-A to control CML cultures on day 5, followed by 30 min incubation and two washes, had no effect on the level of cytotoxicity.

The action of CS-A on the generation of cytotoxic cells, too, was dose-dependent (Fig. 4). CML cultures derived from normal spleen cells incubated with $0.06 \mu\text{g ml}^{-1}$ or more showed little or no cytotoxicity. Higher doses ($0.125 \mu\text{g ml}^{-1}$) were required to obtain significant inhibition of cytotoxicity when

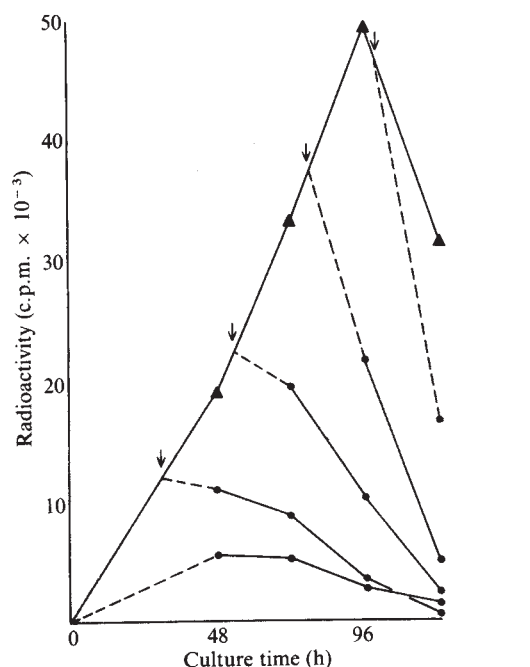


Fig. 1 MLR of mouse spleen cells after addition of CS-A at various times after beginning of culture. MLR's were performed in microtest plates, with each well containing 5×10^5 CBA responder cells and 5×10^5 irradiated (2000R) BALB/c stimulator cells in 0.2 ml RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 3×10^{-5} M 2-mercaptoethanol, 24 mM sodium bicarbonate, penicillin (1 iu per ml) and streptomycin ($1 \mu\text{g ml}^{-1}$). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 : 95% air and pulsed with $1 \mu\text{Ci } ^3\text{H-Tdr}$ at $370 \text{ mCi mmol}^{-1}$ 18 h before the cells were collected. CS-A was dissolved in ethanol and added to the cultures at different times (indicated by arrows) to give a final concentration of $1 \mu\text{g ml}^{-1}$ of CS-A and 0.5% ethanol. The addition of ethanol only did not affect the MLR (data not shown). Δ , Control; $\bullet$, experimental curves after addition of CS-A; $\square$, broken lines, postulate of curves after addition of CS-A.

using cells from presensitized donors, and even a tenfold increase ($1 \mu\text{g ml}^{-1}$) failed to suppress the response completely, despite the fact that at this higher concentration the response of presensitized lymph node cells had been abolished (see Fig. 3). This, together with the sharp break in the curve for presensitized cells of Fig. 4, strongly suggests that there are at least two subpopulations of cytotoxic lymphocytes, with differing sensitivities to the drug. That preformed cytotoxic cells are less susceptible to CS-A suppression has already been shown above

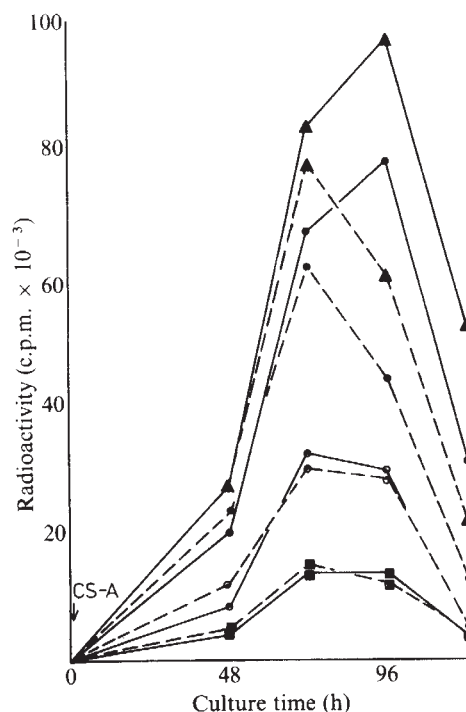


Fig. 2 Addition of different doses of CS-A to cultures of normal or presensitized CBA lymph node cells and DBA/2 stimulator spleen cells. CS-A was added at the beginning of culture. For conditions of culture, see legend to Fig. 1. Solid lines, normal cells; broken lines, presensitized cells; Δ , controls; $\bullet$, $0.05 \mu\text{g ml}^{-1}$; $\square$, $0.125 \mu\text{g ml}^{-1}$; $\diamond$, $0.25 \mu\text{g ml}^{-1}$.

and it is therefore to be assumed that the drug-resistant subpopulation consists of preformed cytotoxic cells present in the spleens of mice sensitized *in vivo*. We have not, however, looked directly for the presence of cytotoxic effector cells in the spleens of *in vivo* sensitized mice, and others¹² have failed to find them 4 weeks after skin graft sensitization, albeit in a different strain combination. It should also be said that while it is reasonable to assume that the cells inhibited at the proliferative stage of MLR are cytotoxic precursors, we cannot exclude the possibility that an entirely different subpopulation of cells, such as T helper lymphocytes, is involved.

These results suggest that higher concentrations of CS-A would be required *in vivo* to suppress T-dependent immune responses in presensitized animals, and preliminary results of skin grafting experiments are consistent with this prediction.

Our MLR data confirm the reports of others^{13,14} who have shown that CS-A suppresses the response of lymphocytes to mitogens such as phytohaemagglutinin and to histocompatibility antigens in MLR. We have shown, further, that the drug is a potent inhibitor of CML provided that it is added in the first 48 h of culture. The similarity between the concentration of CS-A required to inhibit CML and MLR suggests that the suppression of CML is brought about, essentially, by inhibition of the proliferative responses required for the generation of cytotoxic cells; it also provides an explanation for the residual cytotoxic activity in presensitized spleen CML's.

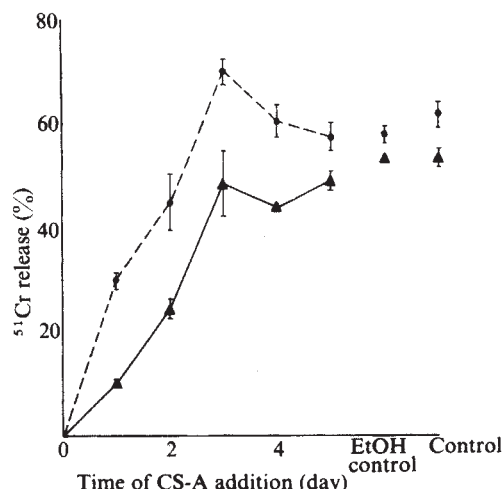


Fig. 3 Addition of CS-A at different times during the course of CML culture between normal or presensitized CBA lymph node cells and DBA/2 stimulator spleen cells: effect on generation of cytotoxicity. CBA responder cells were incubated with irradiated DBA/2 stimulator spleen cells in a ratio of either 1:2 or 2:1 in 10 ml RPMI medium supplemented as described in the legend to Fig. 1, at a total concentration of $3.75 \times 10^6 \text{ ml}^{-1}$. CS-A was dissolved in ethanol and added either at the beginning of the culture or during the course of the next 5 d, to a final concentration of $1 \mu\text{g ml}^{-1}$. The 25 ml flasks were incubated upright at 37°C in a humidified atmosphere of 5% CO_2 :95% air. The cells were collected after 5 d and tested for cytotoxicity in a 4 h ^{51}Cr release assay. Equal volumes (100 μl) of effector cells (at $4 \times 10^6 \text{ ml}^{-1}$) and ^{51}Cr -labelled PY815 mastocytoma target cells (at 10^5 ml^{-1}) were incubated in microtest plates for 4 h at 37°C . The plates were spun at 200g for 5 min, and 100 μl of supernatant from each well was counted. Cytotoxicity (%) was calculated according to the formula:

$$\left(\frac{\text{Test} - \text{background}}{\text{Total release} - \text{background}} \right) \times 100 \text{ (c.p.m.)}$$

Background release of radioactivity from the target cells was never more than 10% and usually less than 5% of the total amount incorporated. ▲, Normal; ●, presensitized. Error bars indicate $\pm 1 \text{ s.e.m.}$

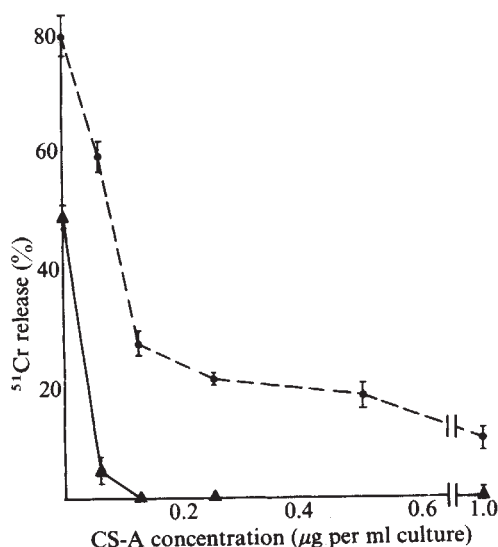


Fig. 4 Addition of different doses of CS-A to CML cultures of either 25×10^6 normal or presensitized CBA spleen cells and 12.5×10^6 DBA/2 stimulator spleen cells: effect on generation of cytotoxicity. CS-A was added at the beginning of culture. For other details, see legend to Fig. 3. Cytotoxicity assays were carried out after 5 d of culture. ▲, Normal; ●, presensitized. Error bars indicate $\pm 1 \text{ s.e.m.}$

CS-A may therefore not be an ideal agent for the reversal of rejection crises in clinical transplantation or in the treatment of presensitized graft recipients.

We thank Miss G. Kalsi for technical assistance and J. F. Borel, Sandoz Ltd, Basle for a gift of cyclosporin A. This work was supported by the Medical Research Council.

Received 28 March; accepted 6 June 1980.

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Endotoxin-induced uveitis in rats as a model for human disease

J. T. Rosenbaum, H. O. McDevitt, R. B. Guss & P. R. Egbert

Department of Medicine, Stanford University School of Medicine, Stanford University Medical Center, Stanford, California 94305

Acute anterior uveitis is a relatively common disease of uncertain aetiology. Its association with a wide variety of systemic disorders including ankylosing spondylitis, Reiter's syndrome, Behcet's disease, juvenile rheumatoid arthritis, psoriatic arthritis, inflammatory bowel disease and sarcoidosis and the prevalence of the HLA antigen B27 (ref. 1) in cases without associated disease have been noted but not explained. In general, animal models for uveitis have not clarified the mechanism of human disease. Most require direct intravitreal injection² or repeated immunizations with retinal or uveal antigens³. Uveitis does occur in adjuvant arthritis, a syndrome that can be produced in rats by the systemic injection of peptidoglycan from the cell wall of a variety of Gram-positive bacteria⁴. The disease, which was initially described following the injection of mycobacteria in mineral oil, has many similarities to Reiter's syndrome⁵, including the development of eye pathology in as many as 40% of the animals⁶. As Reiter's syndrome is associated with enteric Gram-negative infections such as *Shigella*, *Salmonella* and *Yersinia*, we sought to produce an arthritis in rats by injecting killed Gram-negative bacteria. Although we have not observed clinical arthritis in these animals, careful histological study has shown the near-universal appearance of uveitis. The studies described below indicate that the eye disease is due to a portion of the bacterial cell wall known as endotoxin or lipopolysaccharide (LPS).

The typical eye histology produced is shown in Fig. 1. This response could be produced by 1 mg of *Escherichia coli*, *Klebsiella* or *Shigella* but not by 1 mg of *Staphylococcus* or by 0.1 mg of *E. coli*. The different response following Gram-negative as opposed to Gram-positive bacteria encouraged us to assess the reaction to endotoxin alone.

In Lewis rats, we were able to induce uveitis by intravenous or intraperitoneal (i.p.) injections of LPS as well as LPS by the footpad route (i.p.). Comparable abnormal histology was also noted when the source of LPS was extracted from *Shigella flexneri* or *Shigella sonnei* by the Westphal method. Preliminary studies show that the lipid A portion of LPS as obtained by acid hydrolysis and solubilized by coupling to bovine serum albumin⁸ could itself induce uveitis.

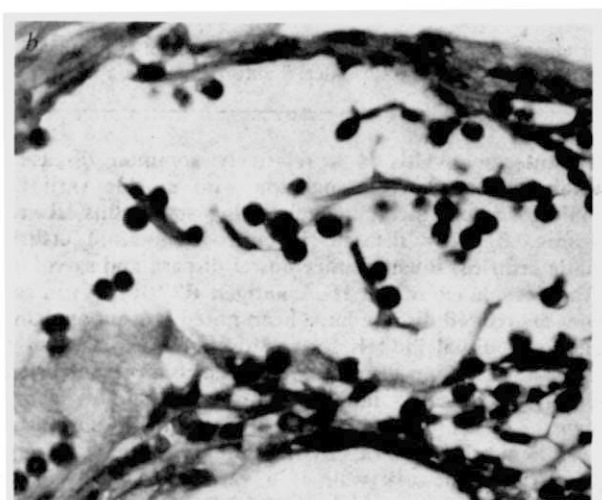
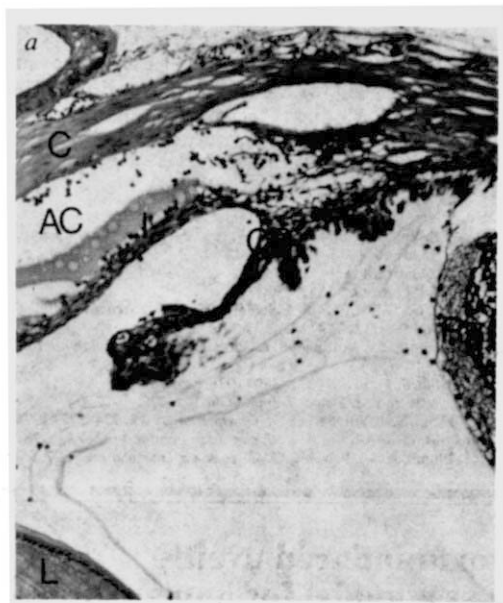


Fig. 1 The top photomicrograph shows many inflammatory cells near the iris (I), anterior chamber (AC) and ciliary body (CB) of a rat eye. A marked proteinaceous exudate is present in the anterior chamber adjacent to the iris. The cornea (C), retina (R) and lens (L) are also labelled for orientation. The photomicrograph below is a higher-power view showing that the infiltrating cells are both polymorphonuclear leukocytes and mononuclear cells. Lewis rats (~250 g), usually 4–6 months old, were used. Bacteria were grown overnight in thioglycollate broth, killed with formalin, washed three times with saline, and lyophilized. Bacteria included *E. coli*, *Klebsiella*, *S. sonnei* and *S. aureus*, coagulase positive. Bacteria were injected with 0.1 cm³ of a mineral oil suspension aseptically into the footpad of a rat that had been lightly anaesthetized with ether. Eyes were obtained 48 h later, after the animal had been killed with ether and exsanguination. Eyes were fixed in 10% neutral buffered formalin and stained with haematoxylin and eosin. Eye pathology was evaluated by two of us (R.G. and P.E.) without knowledge of the treatment received. Eyes were considered to demonstrate uveitis only if a representative cross-section contained five or more inflammatory cells, either polymorphonuclear leukocytes or lymphocytes, in the anterior chamber, posterior chamber or vitreous. In most abnormal eyes a proteinaceous exudate was also apparent. This definition of disease permitted complete concordance by the two observers.

A dose-response curve showed that no detectable changes were present 48 h after 10 µg of *E. coli* LPS injected f.p. but that 50 µg f.p. or 300 µg i.p. produced reactions comparable to that following 100 µg. A dose of LPS sufficient to produce eye disease induced no significant abnormalities in the heart, lung, liver, kidney, spleen, skin, intestine or marrow as judged by light microscopy.

Eyes studied 1 h after an injection of 100 µg LPS f.p. showed no significant abnormality but by 4 h, cells were beginning to accumulate. The disorder was maximal around 48 h, was still present, although declining, 96 h after the injection, and could not be detected 1 week after the injection. When LPS was administered twice over a 3-week period, the disease recurred, but multiple doses of LPS over several weeks tended to abolish or markedly diminish the disease.

In total, bilateral uveitis was seen in all 23 animals 48 h after either 1 mg of lyophilized Gram-negative bacteria or 100 µg LPS given by a variety of routes, in a variety of strains and from a variety of sources (see Table 1). Although a contaminating substance cannot be completely discounted, the reaction seems to be due to endotoxin, in particular the lipid A portion of endotoxin, as argued by its appearance after various Gram-negative bacteria but not after *Staphylococcus aureus* injection and by its occurrence following injection of LPS from different sources.

The absence of cellular abnormalities in other tissues is surprising but it might be predicted from the work of other investigators who have noted that doses of LPS sufficient to cause death (approximately 5 mg in a rat) produce relatively little histological change⁹. The amelioration of disease after repeated injections is reminiscent of the phenomenon of tolerance to endotoxic shock¹⁰.

The concept that bacterial products might induce uveitis is not a novel one. Woods first described 'poisons' from bacteria "which have a definite action upon the uveal tract in the eye" (ref. 11). Others have used live bacteria, primarily streptococcus, and succeeded in producing uveal changes in a minority of rabbits at variable times after injection¹². For a time, elimination of potential foci of infection such as tonsils or carious teeth was in vogue as a method for treating uveitis¹³. Attention focused specifically on endotoxin when Ayo reported a 'toxic ocular reaction' in rabbits that was ascribed to 'Shwartzman toxins' (ref. 14). This response could include an iridocyclitis but disappeared within 24 h and specifically could not be produced in rats¹⁵. A change in ocular vascular permeability following intravenous endotoxin had been noted within 1 h in the rabbit¹⁶. Others have reported that intravenous endotoxin produces an aqueous cellular response in less than 5% of rabbits, although cells in the uvea are common¹⁷.

Endotoxin-induced disease differs markedly from the eye findings in adjuvant arthritis in that the reaction occurs within 24 h of the immunization whereas eye findings in adjuvant disease never occur before 8 days after injection and are maximal at 10–15 days⁶. Eye disease occurs in only a minority of susceptible animals in adjuvant disease⁶, but seems to be near universal in rats after LPS injection.

Circumstantial evidence suggests that this model might have relevance for human disease. The most obvious link is with Crohn's disease and ulcerative colitis. In both of these, circulating endotoxaemia may develop with active disease¹⁸.

In Reiter's syndrome a variety of Gram-negative dysenteries may provoke arthritis and uveitis⁷. In Behçet's syndrome, bowel pathology could conceivably account for a source of endotoxaemia. As endotoxin is known to release lysozyme¹⁹, the elevated lysozyme levels seen in Crohn's disease²⁰, ulcerative colitis²⁰ and Behçet's disease²¹ as well as in sarcoidosis could be related to endotoxin or a stimulus with endotoxin-like activity. Because uveitis in man is closely linked to the HLA antigen B27 (ref. 22), an important advantage of a rat as opposed to a rabbit model, is that the role of the major histocompatibility complex (MHC) can be explored. The variety of strains susceptible to endotoxin-induced uveitis suggests that the effects of LPS are

Table 1 Variety of strains susceptible to endotoxin-induced uveitis

Strain	No. injected with LPS/ no. with uveitis	No. of controls/ no. with uveitis
Lewis	3/3	3/0
Wistar-Lewis	2/2	2/0
Wistar	1/1	
Lea-X	1/1	
BC	1/1	
Fischer	1/1	
Buffalo	2/2	2/0
Sprague-Dawley	2/2	2/0
Long Evans	2/2	2/0
ACI	2/2	1/0
Totals	17/17	12/0

Male rats (200–250 g) were injected in the footpad with *E. coli* LPS 055 (Difco) after light ether anaesthesia. The dose was 100 µg in 0.1 cm³ of saline. Control rats with the exception of the Lewis rats were given an injection of sterile saline alone. Eyes were removed 48 h after injection. Methods for histology and criteria for uveitis were identical to that used following the injection of whole bacteria. Disease was bilateral in all cases.

not restricted by the MHC. However, if the claimed cross-reaction between *Klebsiella* and HLA-B27 is confirmed^{23,24}, B27-positive individuals might be predisposed to endotoxaemia through either mimicry or a receptor²⁵ phenomenon for a *Klebsiella* product. This putative cross-reaction with a Gram-negative bacteria might explain the frequency with which *Klebsiella* is found in the stool of ankylosing spondylitis patients before a flare of uveitis²⁶.

An alternative explanation for the relationship between the MHC and uveitis is that LPS is only one of several factors responsible for disease in man, with a second factor being determined by an immune response gene within the MHC. For example, intravitreal injection of endotoxin can predispose the eye to immune complex deposition²⁷ and preliminary evidence in our laboratory suggests that systemic endotoxin might also facilitate immune complex deposition in the eye. Such a dual causality model would account for both the observed MHC association and the lack of strain restriction for the endotoxin effects.

Current work in our laboratory is aimed at establishing the mechanism of disease in the animal model, testing the possible relevance of the animal disease to uveitis in man, and exploring the potential role of endotoxin in the induction of arthritis and immune complex uveitis.

We thank Ms Bonnie Paschal and Ms Priscilla Hendricks for technical assistance, and Dr Jon C. Ross for interpreting slides of organs other than the eye. *Shigella* strains were from Dr John Shively, *E. coli* LPS from Drs Stuart Koretz and Samuel Strober, and Lewis rats from Simonsen Laboratories, Microbiological Associates or the breeding colony of Dr Irving Weissman.

Received 26 December 1979; accepted 3 June 1980.

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Elution of herpes simplex virus-specific cytotoxic antibodies from squamous cell carcinoma of uterine cervix

Pradeep Seth & N. Balachandran

Department of Microbiology,
All India Institute of Medical Sciences,
Ansari nagar, New Delhi-16, India

There is evidence that carcinoma of the cervix uteri is associated with infection with herpes simplex virus type 2 (HSV-2), and although indirect evidence suggests an aetiological relationship^{1–6}, this remains controversial^{7–9}. One facet of the problem is that complement-dependent cytotoxic antibodies to HSV-2-infected cells have been undetectable or found only in low titre in sera from patients with progressive cervical cancer whereas higher titres have been found where cervical lesions are less advanced^{10,11}. Indeed, cytotoxic antibody titres have been found to rise during the follow-up period after successful treatment¹¹. Absorption of these antibodies by the tumour cells has been proposed as a possible reason for low HSV-2 cytolytic reactivity in patients with progressing cervical cancer¹⁰. This explanation assumes the presence of HSV-2-specific antigen(s) on the tumour cells. We now provide support for this assumption with evidence that absorbed HSV-2-specific antibodies can be eluted from cervical cancer tissue.

Elution of bound immunoglobulin from freshly removed tissues from six patients with cervical cancer, stage I (five patients with squamous cell carcinoma, one with adenocarcinoma), one patient with ovarian cancer, two patients with uterine fibroids, three patients with squamous cell carcinoma of the skin and from three normal uterine cervixes was achieved by treatment with 15% NaCl (pH 7.4) at 37 °C (ref. 12). The protein content of the eluates was estimated¹³ and the class of immunoglobulin present was determined by polyacrylamide gel electrophoresis using purified human serum IgM, IgG and IgA as markers. Although the eluates from all tissues contained proteins, IgG was the only detectable immunoglobulin eluted from all squamous cell carcinoma tissues as well as cervical adenocarcinoma tissues (Table 1). Tissues from uterine fibroids and normal cervix failed to yield any immunoglobulin by the technique used. The nature of other protein(s) present in the eluates was not determined.

Complement-dependent cytolytic activity of the eluate was assayed on ⁵¹Cr-labelled BHK-21 cells infected 22–24 h previously with HSV-1 or HSV-2, at a multiplicity of infection of 3–5 plaque forming units (PFU) per cell¹⁴. The eluate (0.1 ml) was diluted 1:5 in Tris-buffered saline, pH 7.2, mixed with 5 × 10⁴ cells and incubated at 37 °C for 60 min. Then, 0.2 ml of fresh guinea pig complement diluted 1:10 was added and the mixture was incubated at 37 °C for another 90 min. The cytotoxicity was stopped by adding 2 ml of cold Tris-diluent to each tube, which was then centrifuged at 600g for 3 min at 4 °C. Aliquots (1 ml) of the supernatant were transferred to another tube and counted for radioactivity in a Packard automatic γ spectrometer. Each test included: labelled cells only, which were frozen and thawed in distilled water for maximum ⁵¹Cr release, cells with eluate alone to control for eluate toxicity, and cells

Table 1 Complement-dependent antibody-mediated lysis of infected (HSV, vaccinia) and uninfected BHK-21 cells by the eluates from cancer and normal tissues

Tissue processed		Lysis by the eluate* of ⁵¹ Cr-labelled BHK-21 cells							
Source	Weight (g)	Protein conc. of eluate (ng ml ⁻¹)	Class of Ig present in eluate	Virus infecting BHK-21 cells	Maximum release control (c.p.m.)	Spontaneous release control (c.p.m.)	Eluate toxicity control (c.p.m.)†	Test release (c.p.m.)	% Specific release‡
Squamous cell cancer of cervix stage I	2.045	300	IgG	HSV-1	2,365	278	139	1,655	66
				HSV-2	2,476	369	147	2,471	100
				Vaccinia	3,565	581	350	397	0
				None	3,972	307	152	187	0
Squamous cell cancer of cervix stage I	1.80	111	IgG	HSV-1	2,365	278	172	487	10
				HSV-2	2,476	369	185	685	15
				Vaccinia	3,565	581	337	382	0
				None	3,972	307	147	182	0
Squamous cell cancer of cervix, stage I	1.40	83	IgG	HSV-1	2,365	278	181	491	10
				HSV-2	2,476	369	177	643	13
				Vaccinia	3,565	581	386	453	0
				None	3,972	307	175	208	0
Squamous cell cancer of cervix, stage I	0.630	233	IgG	HSV-1	1,973	205	114	346	8
				HSV-2	1,791	211	127	371	10
				Vaccinia	3,565	581	275	346	0
				None	3,972	307	182	211	0
Squamous cell cancer of cervix, stage I	2.255	620	IgG	HSV-1	1,973	205	121	435	13
				HSV-2	1,791	211	113	451	15
				Vaccinia	3,565	581	285	369	0
				None	3,972	307	197	218	0
Squamous cell cancer of skin	1.90	120	IgG	HSV-1	2,789	383	211	219	0
				HSV-2	2,561	367	207	251	0
				Vaccinia	2,856	309	259	281	0
				None	3,331	218	101	123	0
Squamous cell cancer of skin	2.35	185	IgG	HSV-1	2,789	383	195	238	0
				HSV-2	2,561	367	219	227	0
				Vaccinia	2,856	309	272	285	0
				None	3,331	218	111	139	0
Squamous cell cancer of skin	2.13	163	IgG	HSV-1	2,789	383	203	213	0
				HSV-2	2,561	367	189	256	0
				Vaccinia	2,856	309	223	249	0
				None	3,331	218	137	148	0
Adenocarcinoma of cervix, stage I	2.125	244	IgG	HSV-1	2,365	278	141	173	0
				HSV-2	2,476	369	163	210	0
				Vaccinia	3,565	581	277	381	0
				None	3,972	307	161	201	0
Ovarian carcinoma	1.95	ND	ND	HSV-1	1,973	205	179	191	0
				HSV-2	1,791	211	151	177	0
				Vaccinia	3,565	581	309	403	0
				None	3,972	307	153	192	0
Fibroid uterus	4.010	540	Nil	HSV-1	2,365	278	143	201	0
				HSV-2	2,476	369	147	258	0
				Vaccinia	3,565	581	367	411	0
				None	3,972	307	148	207	0
Fibroid uterus	1.41	140	Nil	HSV-1	2,365	278	137	161	0
				HSV-2	2,476	369	151	207	0
				Vaccinia	3,565	581	352	275	0
				none	3,972	307	129	192	0
Normal cervix	1.13	540	Nil	HSV-1	1,973	205	127	141	0
				HSV-2	1,791	211	146	179	0
				Vaccinia	3,565	581	321	238	0
				None	3,972	307	208	222	0
Normal cervix	2.22	80	Nil	HSV-1	1,973	205	141	159	0
				HSV-2	1,791	211	110	143	0
				Vaccinia	3,565	581	357	259	0
				None	3,972	307	179	215	0
Normal cervix	1.85	190	Nil	HSV-1	1,973	205	136	147	0
				HSV-2	1,791	211	161	169	0
				Vaccinia	3,565	581	283	197	0
				None	3,972	307	182	229	0

Each tissue was minced finely and passed through a 60-wire mesh screen to obtain a cell suspension. It was washed 15 times with 10-ml volumes of phosphate-buffered saline (0.025 M PBS, pH 7.4) and the UV absorbance of the supernatant after the 15th wash was checked at 280 nm wavelength in a spectrometer. No further washing was done if *A* was less than 0.025. When it was more, additional washings were given until *A* was less than 0.025. The pellet was then suspended in 5 ml of 15% NaCl (pH 7.4) and incubated at 37 °C for 2 h. After that, it was centrifuged at 10,000g for 10 min, and the sediment was re-extracted with 15% NaCl. The supernatants of both extractions were pooled and dialysed against 500 ml of PBS for 24 h with two changes of buffer. It was then centrifuged at 36,000g for 1 h and the supernatant was collected and concentrated 10-fold by vacuum dialysis. It was centrifuged at 5,000g for 30 min and the clear supernatant was taken as eluate for further testing as described in the text. ND, Not done.

* A 1:5 dilution of the eluate was tested.

‡ % Specific release = $\frac{\text{test release (c.p.m.)} - \text{spontaneous release (c.p.m.)}}{\text{maximum release (c.p.m.)} - \text{spontaneous release (c.p.m.)}} \times 100$.

† Values of eluate toxicity controls are always less than spontaneous release control. In cases where the values of the former are greater than the latter, the eluate is too toxic to be tested.

with complement alone as a control for spontaneous ^{51}Cr release. All test systems, including controls, were carried out in duplicate and their c.p.m. were averaged. The cytolytic antibody activity was expressed as the per cent specific release of ^{51}Cr according to the formula¹⁵

$$\% \text{ Specific release} = \left(\frac{{}^{51}\text{Cr release in the presence of eluate and complement} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \right) \times 100$$

The eluates from cervical squamous cell carcinoma produced 8–66% lysis of HSV-1 infected cells and 10–100% lysis of HSV-2-infected cells (see Table 1). The cytolytic pattern of the eluates suggests the presence of antibody activity to cross-reacting antigens of HSV-1 and HSV-2. However, the cytolytic activity was more pronounced against HSV-2-infected cells. Specificity of the antibody activity was established by the failure of the eluates to lyse vaccinia-infected BHK-21 cells¹⁶ or uninfected BHK-21 cells. Moreover, eluates from other tumour tissues as well as normal healthy cervix did not show any cytolytic activity against infected or uninfected BHK-21 cells.

Each tissue was also tested for the presence of virus. A portion of tissue was ground in a homogenizer and 0.1 ml of the homogenate was inoculated onto two stationary cultures of Vero cells grown in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum. The cultures were incubated at 37°C and observed for 14 days. None of the homogenates produced a cytopathic effect suggestive of HSV. Furthermore, three tissues from squamous cell carcinoma of the cervix, three tissues from squamous cell carcinoma of the skin, one tissue each from adenocarcinoma of the cervix, uterine fibroid and normal cervix were minced separately into 1-mm³ explants and co-cultivated with monolayers of Vero cells which were then fed with MEM containing 20% fetal calf serum¹⁷. The cultures were observed for 8 weeks for cytopathic effects before they were discarded. No virus was recovered on co-cultivation.

These data indicate the presence of HSV-specific antigens on the tumour cells of squamous cell carcinoma of the uterine cervix in the absence of apparent infection of these cells by the virus. However, latent or abortive infection of the tumour tissue by HSV cannot be definitely excluded, even though no HSV was isolated from any cervical cancer tissues.

Evidently, HSV-specific cytotoxic antibodies had been absorbed from the blood of the patients with cervical cancer *in vivo* by HSV-specific antigens present on the tumour cells. This absorption seems to be specific for squamous cell carcinoma of the cervix, as HSV-specific immunoglobulins could not be eluted from squamous cell carcinoma of the skin. The absorbed antibody may be either functioning as blocking antibody in tumour enhancement¹⁸ or involved in the cell-mediated destruction of the tumour cells¹⁹. Furthermore, the absence of HSV-specific antigens in tissues from squamous cell carcinoma of the skin, ovarian carcinoma and uterine fibroids strongly suggests a role of HSV in the aetiology of squamous cell carcinoma of the uterine cervix.

On the basis of the present data, it is difficult to understand how the low titres of HSV-cytolytic antibodies in the sera of patients with progressing cancer of the cervix could arise from absorption of antibodies by the tumour cells¹⁰. However, the presence of circulating immune complexes in the sera of patients with cervical cancer²⁰ might interfere with the assay of cytolytic antibodies by the ^{51}Cr -release test, and absorption of antibodies by the tumour cells may account for depletion of only a small portion of cytolytic antibodies from the circulation.

These results, although obtained using only a few patients, are nevertheless striking. As no infective virus could be detected or reactivated, it is tempting to suggest that the virus-specific IgG is adsorbed to virus-specified determinants on the neoplastic cells.

We thank Drs V. L. Bhargava, S. Kumar and D. Ghosh for the clinical material and Drs L. N. Mohapatra, S. Balaya and R. Kumar for their advice.

Received 9 April; accepted 13 June 1980.

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How many types of erythroleukaemia are induced by retroviruses in mice?

Rita Anand & Richard Steeves*

Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Erythroleukaemia, until now defined by morphological criteria, has been observed in mice infected with several distinct virological entities, including the polycythaemia-inducing strains of Friend virus complex (FV-P)^{1,2}, the anaemia-inducing strains of Friend³ and Rauscher^{4,5} virus complex (FV-A and RV-A, respectively) and various helper-independent virus isolates derived from the FV-A⁶ or RV-A⁷ complexes. Whereas erythroleukaemia develops rapidly (within 2–3 weeks) in mice infected with any strain of FV or RV, the helper-independent virus alone is pathogenic in mice only if they are infected neonatally^{6,7}. We now describe how two biological markers can be used to distinguish among the otherwise confusing array of virus-induced erythroleukaemias in mice. The evidence suggests that there are three different classes of this disease: (1) those (S⁺E⁺) from which both defective spleen focus-forming virus (SFFV) and erythropoietin-independent, erythroid colony-forming units (CFU-EI) can be recovered, (2) those (S⁺E⁻) from which only SFFV (but not CFU-EI) can be recovered, and (3) those (S⁻E⁻) from which neither SFFV nor CFU-EI can be recovered. There is a consistent association between the type of virus used to induce the erythroleukaemia and the class of the disease induced.

Since Friend's isolation of a virus that rapidly induced "a disease having the character of a leukaemia" (ref. 8), there have been many reports on Friend virus, some containing different interpretations of the disease induced by this virus^{3,9,10}, others describing different host responses to different strains of the virus^{11,12}, and still others describing different isolations of similar viruses which induce diseases similar to that induced by Friend virus^{6,7,13–16}. About a decade ago a defective virus in Friend^{17,18} and Rauscher¹⁹ virus preparations was discovered which has the capacity to induce macroscopic spleen foci^{20,21} and sometimes polycythaemia¹² in susceptible strains of mice. Clearer knowledge of the virus came from Troxler and colleagues^{6,22–24}, who analysed single cell clones inoculated with FV-P or with FV-A, and developed from each type of infected clone SFFV-infected non-producer cell lines that were free of detectable helper virus. When they used different helper viruses to rescue infectious pseudotypes of SFFV, they found that the haematocrit response of infected hosts was associated with the origin of SFFV (from FV-P- or FV-A-infected cells) rather than with that of its helper virus²⁴.

* Present address: Division of Radiation Oncology, University of Wisconsin, 600 Highland Avenue, Madison, Wisconsin 53792.

Table 1 Three host response patterns to erythroleukaemia-inducing retroviruses

Virus strain	Recovery of SFFV ($\times 10^3$ FFU ml $^{-1}$)	Recovery of CFU-EI (per 10^5 spleen cells)
FV-P (Lilly)	52.0	620
FV-P (Axelrad)	78.4	371
FV-P (Mirand)	0.2	85
FV-A (Dmochowski)	0.5	0
RV-A (Rauscher)	3.5	0
F-MuLV ₂₀₁	0	0
RV-A variant (Fredrickson)	0	0

Abbreviations: CFU-EI, erythropoietin-independent, erythroid colony-forming unit; FV-P, polycythaemia-inducing strain of Friend virus complex; FV-A, anaemia-inducing strain of Friend virus complex; F-MuLV₂₀₁, clone 201 of a helper-independent murine leukaemia virus (MuLV) that was isolated from FV-A²⁴; RV-A, anaemia-inducing strain of Rauscher virus complex; RV-A variant, helper-independent MuLV isolated from RV-A that lacks spleen focus-forming virus (SFFV)⁷. Virus complexes (the first five strains) considered to contain SFFV were diluted 10^{-1} from stock (10% w/v spleen homogenates) and injected intravenously into young adult BALB/c mice. Two weeks later their spleens were collected and assayed for SFFV by the spleen focus assay²⁰ and for CFU-EI according to the method of Liao and Axelrad²⁷. Helper-independent MuLVs (the last two strains) considered to lack SFFV were not diluted from stock, but were injected intraperitoneally into suckling BALB/c mice within 48 h of birth. Three to four weeks later their spleens were collected and assayed as described above.

However, the pathology of erythroid leukaemias and dyscrasias in mice has not been well defined, and there has been little agreement among the morphological descriptions of virus-induced diseases of this sort. For example some investigators have described the diseases induced by the same viruses as erythroblastosis^{9,10,25,26} or simply erythrocytopoietic disease¹³. In a search for non-morphological criteria that could be used to define erythroid leukaemias, we compared several different virus strains according to their capacity to initiate SFFV replication and/or the production of CFU-EI²⁷ in BALB/c mice. The results of this comparison, summarized in Table 1, show that by 3 weeks after infection the diseases induced could be divided into three categories on the basis of the recovery from splenic tissues of: both SFFV and CFU-EI (S^+E^+); SFFV but not CFU-EI (S^+E^-); neither SFFV nor CFU-EI (S^-E^-).

Let us now correlate these classes of erythroid leukaemia with the viruses used to induce them. Because of previous reports^{12,27} it was not surprising that all strains of FV-P would induce S^+E^+ leukaemias. (On the basis of this clear association we would predict that the recently discovered, cloned, murine sarcoma virus²⁸ that induces an S^+E^+ erythroid cell proliferation might also induce polycythaemia.) The presence of SFFV in FV-A-infected mice was also not surprising, for earlier conflicting reports on this finding^{12,29} were recently settled by the elegant cloning experiments of Troxler *et al.*²⁴. However, the absence of CFU-EI among SFFV-positive leukaemia cells of FV-A-infected mice was significant.

Could the lack of CFU-EI in FV-A-infected mice be attributed to the lower titres of SFFV usually encountered in FV-A stocks³⁰? Probably not, as the recovery of SFFV from mice infected with a low dose of FV-P (Mirand) was 10-fold lower than that of FV-A-infected mice (Table 1), although CFU-EI were only recovered from the former. Also, Steinheider *et al.*³¹ failed to recover CFU-EI from FV-A-infected DBA/2 mice given an amount of SFFV 'comparable' to that given to CFU-EI-positive, FV-P-infected mice, although the actual dose of SFFV in the infecting inoculum was not stated. In the present study (data not shown) CFU-EI were readily detected in mice after infection with FV-P diluted almost to the end point for

SFFV. Therefore, it seems that CFU-EI are not an expression of SFFV infection *per se*, but of erythropoietin-independent erythroid precursors destined to contribute to the development of polycythaemia. Their apparent absence after FV-A infection is in keeping with the failure of infected hosts to develop polycythaemia; this provides another qualitative distinction (in addition to the haematocrit response) between FV-A and FV-P.

The last (S^-E^-) class of leukaemias, like the first two classes, is distinctly erythroid in nature^{22,32}, and disease can be induced almost as rapidly (within 3–4 weeks of infection). Unlike the first two classes, the S^-E^- leukaemias develop only if the recipient mice have been inoculated with virus neonatally, probably due to the lack of immunodepressive SFFV in the viral inocula. This absence of SFFV was clearly shown for F-MuLV clone 201 by transfection experiments with molecularly cloned viral DNA³³. Even though mice infected neonatally with F-MuLV₂₀₁ or with the Fredrickson variant of RV-A⁷ did not yield SFFV or CFU-EI (Table 1), they did develop erythroid leukaemia, as tested by cytological examination of blood smears or splenic imprints.

Other candidate viral agents that might induce S^-E^- leukaemias are Dawson's F-MuLV variant¹⁶ and Kirsten's MuLV¹⁴. Unfortunately, the former was not available to us, and samples of the latter failed to induce disease in our mice. There are other examples of murine viruses, apparently lacking SFFV, with the capacity to induce leukaemia³⁴. These agents should all be examined and compared with the prototype strain, F-MuLV₂₀₁, by both biological and biochemical means.

It is curious that F-MuLV₂₀₁, isolated from FV-A and regularly collected from the spleens of infected mice only 3 weeks post-infection, should induce erythroid leukaemia, whereas F-MuLV originally isolated from the Friend or Rauscher virus complexes induced lymphoid leukaemias^{35–37}. The latter viruses were obtained from lymphomatous lymph nodes and spleen 3–4 months post-infection, so it is possible that differences in the passage histories of these viruses altered their tissue tropism. Alternatively, there may be a genetic difference between erythrotropic and lymphotropic forms of F-MuLV in Friend and Rauscher virus stocks. One way to test the latter possibility might be to compare the pathogenic properties of erythrotropic and lymphotropic MuLV pseudotypes of polycythaemia-inducing and anaemia-inducing strains of SFFV. Such a study, recently made by Troxler *et al.*²⁴ with erythrotropic F-MuLV₂₀₁ and lymphotropic Moloney MuLV, failed to show any effect of the pseudotyping helper virus on the haematocrit response of mice to either polycythaemia-inducing SFFV or anaemia-inducing SFFV. More studies are needed to clarify the pathogenic roles of helper virus and defective virus in the induction of erythroleukaemia.

We thank Dr Frank Lilly for helpful discussions and Mrs Ida Shapiro and Mr Vernon Grant for secretarial and technical assistance, respectively. This investigation was supported by NCI grant CA-19873.

Received 12 March; accepted 5 June 1980.

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New regulators of melanin biosynthesis and the autodestruction of melanoma cells

John Pawelek*, Ann Körner, Alan Bergstrom & Jean Bologna

Department of Dermatology, Yale University School of Medicine, New Haven, Connecticut 06510

The hues of mammalian skin and hair vary from white to jet black. Silvers has recently catalogued over 130 different coat colour mutations at more than 50 genetic loci in the house mouse (*Mus musculus*), a useful model for study¹. Some of these loci govern differentiation of melanocytes and their migration from the neural crest region during embryogenesis². Others, however, involve expression of genes for melanin synthesis within the pigment cell, such as *c*-locus mutations, where animals are phenotypically albino. *c*-locus albinism is presumably due to a deficiency of active tyrosinase, the enzyme catalysing the initial steps in the melanin synthetic pathway. It has generally been assumed that the only regulated steps in this pathway are those governed by tyrosinase, since *in vitro*, melanin is spontaneously formed from dopa quinone (Fig. 1). However, Logan and Weatherhead have provided evidence for post-tyrosinase inhibition of melanogenesis by melatonin in hair follicles of Siberian hamsters³, as do our studies with various mutants of Cloudman mouse melanoma cells, suggesting the involvement of a second regulatory site. We describe here three new factors which control melanin synthesis that we have partially purified from Cloudman cells: (1) dopachrome conversion factor (DCF), which accelerates the conversion of dopachrome to 5,6-dihydroxyindole (see ref. 6 for detailed description); (2) 5,6-dihydroxyindole conversion factor, which catalyses the conversion of 5,6-dihydroxyindole to melanin and is active only when cells are exposed to melanotropin (MSH), and (3) 5,6-dihydroxyindole blocking factor, which restricts melanogenesis at 5,6-dihydroxyindole. This third factor appears to protect cells from the cytotoxic effects of melanin precursors, and it is removed when the cells are exposed to MSH. Discovery of the factors indicates that regulation of the melanin biosynthetic pathway is more complex than previously supposed.

DCF has recently been described⁶. In brief, it was first discovered by the observation that extracts of amelanotic melanoma cells rapidly converted dopachrome to a colourless compound. A factor was subsequently isolated that catalysed the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid followed by a spontaneous decarboxylation to

5,6-dihydroxyindole. DCF reacts specifically with dopachrome, having no effect on tyrosine or dopa. DCF was found in two different mouse melanomas (B16 and Cloudman S91), the hamster melanoma of Greene⁷, and mushrooms. It was absent from Friend erythroleukaemia cells, neuroblastoma cells, myeloma cells, and L-cell fibroblasts. Serial dilutions of melanoma cell extracts showed that equal amounts of DCF were present in cells whether or not they had been exposed to MSH. All three factors were observed in experiments described below. Extracts of control cells or MSH-treated cells were mixed with dopachrome. In each case there was a rapid decrease in absorbance at 475 nm due to DCF activity compared with the slow, spontaneous decrease in the buffer blank (Fig. 2a). (Note that this figure shows the DCF reaction in extracts of control cells. The extent and magnitude of the reactions were the same in extracts of MSH-treated cells.) Following the DCF reaction, the samples containing extracts of control cells remained colourless for at least 24 h. However, samples containing extracts of MSH-treated cells turned dark rapidly as melanochrome (absorbing at 540 nm) and then melanin were formed (Fig. 2b). Melanin production in reactions with extracts of MSH-treated cells was faster than that occurring spontaneously in the reactions containing only buffer and dopachrome.

These experiments were extended using 5,6-dihydroxyindole rather than dopachrome as a substrate. Extracts of control or MSH-treated cells were mixed with 5,6-dihydroxyindole for 3 h at 30°C. The following points were evident: (1) there was a slow, spontaneous conversion of 5,6-dihydroxyindole to melanin in tubes containing buffer only; (2) melanin production was prevented by extracts of control cells; and (3) melanin production was accelerated by extracts of MSH-treated cells. After 24 h the tube containing buffer had turned black due to spontaneous melanin formation while the tubes containing extract of control cells remained colourless (data not shown).

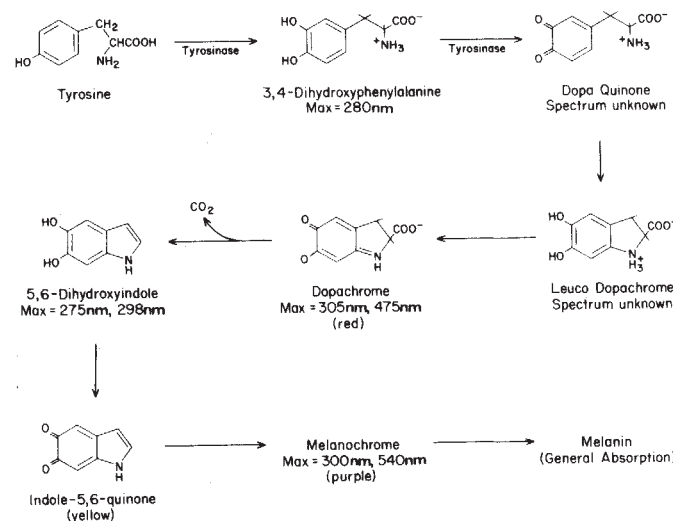


Fig. 1 Stages in the oxidation of tyrosine to melanin, modified slightly from the scheme of Mason and Raper³. Note that while this scheme depicts melanin biosynthesis as a polymerization of indole-5,6-quinone alone, it has been shown that mammalian melanins are heteropolymers, containing many different combinations of the precursors shown⁴.

Samples were removed from the tubes and analysed for 5,6-dihydroxyindole content by HPLC (Fig. 3). The 5,6-dihydroxyindole peaks (arrows) were integrated and the results are as follows. Panel *a* shows the 5,6-dihydroxyindole content of a mixture of buffer and indole that was kept on ice for 3 h. Panel *b* shows 5,6-dihydroxyindole from the same mixture that was incubated for 3 h at 30°C (89% of the sample in *a*). Panel *c* shows the 5,6-dihydroxyindole content of an incubated mixture

* To whom requests for reprints should be sent.

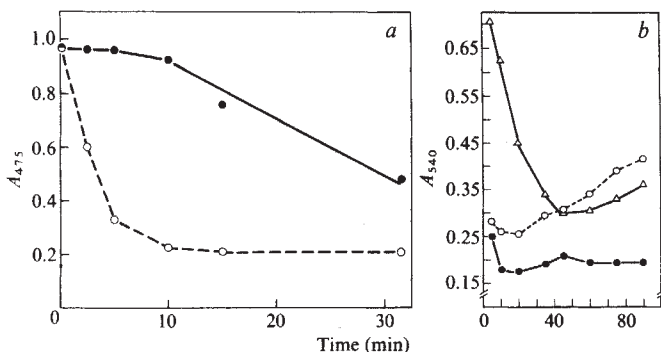


Fig. 2 Effects of cell extracts on dopachrome. Extracts were prepared from an MSH-responsive cell line (PSI-mel-1 (ref. 8)) by the following procedure. Cells were collected with EDTA (1 mM) in isotonic saline, spun for 10 min at 2,000g, and lysed with 5 vol ice-cold Triton X-100 (0.5%) in sodium phosphate buffer (50 mM, pH 6.8). The lysate was then centrifuged (30,000g, 15 min) and the pellet containing melanin and cellular debris was discarded. Protein content of the supernatant fraction was determined by the method of Bradford⁹. Such extracts were prepared from control cells and cells that had been exposed to MSH (2×10^{-7} M) for 3 days (isobutylmethyl xanthine was not used in this experiment). The extracts (0.1 ml, 0.4 mg protein) were mixed with 0.2 ml dopachrome which had been diluted for absorbance measurements. Panel *a* shows a decrease in absorbance due to dopachrome at 475 nm after different incubation periods at room temperature with extracts from control cells (solid line: buffer; dotted line: cell extract). The disappearance in absorbance with time was due to the action of DCF which was present in equal amounts in extracts of both control and MSH-treated cells⁶. Panel *b* shows the changes in absorbance at 540 nm with time. The initial decreases were due to DCF activity since dopachrome shows some absorbance at 540 nm. Increases in absorbance at 540 nm were due to melanin synthesis which could be readily detected by the eye. Dopachrome solution was prepared by mixing a solution of dopa with Ag_2O and filtering¹⁰. ●, Control cell extract; ○, MSH-treated cell extract; △, buffer blank.

of indole and control cell extract (98% of the sample in *a*). Panel *d* shows the 5,6-dihydroxyindole content in the incubated mixture of indole and MSH-treated cell extract (5.7% of the sample in *a*). Thus direct measurements of the 5,6-dihydroxyindole content of the tubes confirmed what was visually apparent: melanin synthesis from 5,6-dihydroxyindole was prevented by control cell extracts and accelerated by MSH-treated cell extracts. These experiments demonstrate the existence of both a 5,6-dihydroxyindole conversion factor and a blocking factor. MSH treatment removes the blocking factor and allows for expression of the conversion factor.

It has been known for some time that melanin precursors can be cytotoxic¹²⁻¹⁵. Because MSH increases tyrosinase levels one would predict that, in the presence of MSH, the rate of production of melanin precursors is also increased. This has been shown to be the case in culture as cells exposed to MSH are much more susceptible to the toxic effects of tyrosine and dopa than control cells¹⁴.

If it were true that only tyrosinase activity were affected by MSH, then the toxicity of melanin precursors distal to dopa quinone should not be influenced by the presence or absence of MSH. We show below that this is not the case. We have found that the toxic effects of dopachrome are markedly increased when the target cells have been treated with MSH (Fig. 4). (Note in Fig. 4 legend that 3-methylisobutylxanthine was used to potentiate the MSH effect.) The effects were quite striking when observed with the phase microscope. After only 1 h exposure to dopachrome (10^{-4} M), cells preincubated with MSH began to lyse. In contrast, cells exposed during this same time to either control culture medium, or medium containing MSH, dopa plus MSH, or dopachrome alone still appeared healthy. About

fivefold less dopachrome was sufficient for 50% killing when the cells were exposed to MSH compared to when they were not exposed to the hormone. Addition of dibutyl-cyclic-AMP (10^{-3} M) also increased the sensitivity to dopachrome, indicating that the cellular response was likely to be cyclic-AMP mediated (data not shown). The dose-response studies shown in Fig. 4 were done in parallel with an amelanotic variant cell line⁸ that showed a very weak response to MSH in terms of pigment production. The 'non-responsive' line was only slightly sensitized by MSH to the toxicity of dopachrome. Extracts of the amelanotic line contained 5,6-dihydroxyindole blocking factor and no 5,6-dihydroxyindole conversion factor activity whether or not the cells were exposed to MSH (data not shown). We feel that the most likely explanation for the increased sensitivity of MSH-activated cells to dopachrome is the removal of the 5,6-dihydroxyindole blocking factor and the expression of active 5,6-dihydroxyindole conversion factor. We realize, however, that our evidence is only circumstantial and that more experiments are necessary to prove these points.

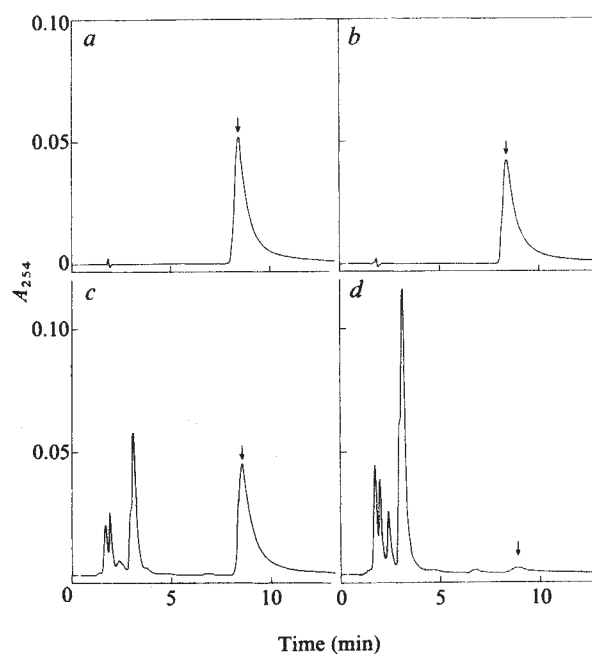


Fig. 3 Effects of extracts prepared from control- and MSH-treated cells on 5,6-dihydroxyindole as analysed by HPLC. Experimental design is outlined in the legend to Fig. 2. 5,6-dihydroxyindole (3.5×10^{-4} M) was used instead of dopachrome. Protein concentrations in the undiluted cell extracts were 7 mg ml^{-1} . The reaction mixes were adjusted to 100 μl with sodium phosphate (0.05 M, pH 6.8), incubated in a shaking water bath at 30°C for 3 h and aliquots (20 μl) from the tubes were analysed for 5,6-dihydroxyindole content. An Altex Model 330 Isocratic liquid chromatograph equipped with a Model 110A pump and a 20- μl sample loop was used. Absorbance of the eluent was monitored at 254 nm with an 8- μl Analytical Optical Unit. Experiments were performed with an Ultrasil ODS Column (Altex Instruments, Inc.), 0.46 cm internal diameter $\times$ 25 cm. The column was packed with octadecyl-silica particles of 10 μm average diameter. The mobile phase was methanol/sodium phosphate (50 mM, pH 6.5) 1:9. Flow rate was maintained at 1.8 ml min^{-1} . 5,6-dihydroxyindole eluted at about 8.4 min (arrows). The indole was synthesized as described by Axelrod and Lerner¹¹ and stored in crystalline form in N_2 at -20°C . Before each experiment, appropriate amounts of 5,6-dihydroxyindole were dissolved in degassed buffer (sodium phosphate, 0.05 M, pH 6.5). *a*, Indole plus buffer, 3 h in ice; *b*, indole plus buffer, 3 h at 30°C ; *c*, indole plus control cell extract (50 μl), 3 h at 30°C ; *d*, indole plus extract (50 μl) of MSH-treated cells, 3 h at 30°C . Material that eluted between 1 and 4 min was from the cell extracts alone and did not change during the 3-h incubation period.

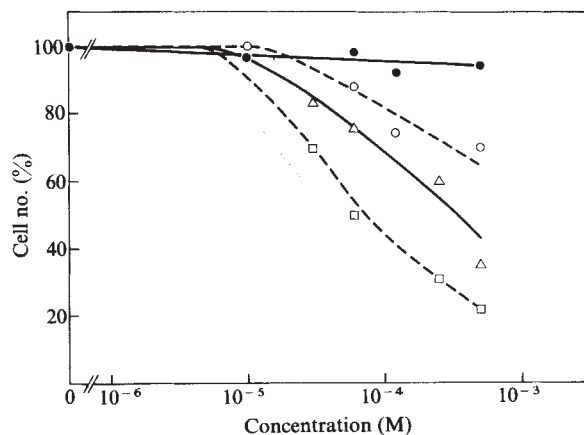


Fig. 4 Effects of MSH on the toxicity of dopa and dopachrome against Cloudman S91 mouse melanoma cells. Cells (10^5) from an MSH-responsive line (PSI-mel-1 (ref. 8)) were cultured in monolayer for two days either in plain culture medium or in medium supplemented with MSH (2×10^{-7} M) and methylisobutylxanthine (MIX 5×10^{-5} M). Two days later the cells were given fresh medium containing dopa or dopachrome as indicated. After 2.5 h, the dopa and dopachrome were removed and the cells were given fresh medium. The next day the cells were collected with EDTA (1 mM) in isotonic saline, and counted in a Coulter counter. Dopachrome was prepared as above by oxidizing dopa with Ag_2O . Control cultures received either buffer that had been mixed with Ag_2O and filtered, or buffer only (sodium phosphate, 0.05 M pH 6.8). The control cell numbers were similar in each case. Values represent the averages from quadruplicate cultures and the variation was less than $\pm 10\%$. The experiments were repeated several times with similar results. MIX, a phosphodiesterase inhibitor, was used to potentiate the MSH effect. At the concentration used (5×10^{-5} M) MIX had no effect by itself. ●, Dopa; ○, dopa + MSH; △, dopachrome; □, dopachrome + MSH.

Transformation-specific secreted phosphoproteins

Donald R. Senger*, Dyann F. Wirth†
& Richard O. Hynes

Department of Biology and Center for Cancer Research,
Massachusetts Institute of Technology, Cambridge,
Massachusetts 02139

We have previously shown that transformed mammalian cells secrete two classes of transformation-specific proteins of molecular weight (MW) about 60,000 (ref. 1). Secretion of both classes of proteins occurs regardless of the transforming agent and is temperature-sensitive in a cell line which is temperature-sensitive for the transformed phenotype (ts B77 Rat 1; see ref. 1). One of the classes consists of non-phosphorylated polypeptides. The proteins of this class, derived from transformed mouse, rat and hamster lines, are all antigenically related to one another. The second class of transformation-specific secreted proteins is antigenically distinct from the first class, that is, it is not precipitated by antiserum which recognizes the non-phosphorylated class. This second class comprises major phosphoproteins also in the 60,000 MW range¹. These major phosphoproteins were found to be secreted by transformed hamster, rat, mouse and rabbit cells. We now report that these transformation-specific phosphoproteins are all antigenically related, regardless of the transforming agent. The phosphoproteins are synthesized by the cells, phosphorylated intracellularly and subsequently released into the culture medium. These transformed cells also secrete protein kinase activities capable of phosphorylating the transformation-specific phosphoproteins *in vitro*. The major sites of *in vivo* and *in vitro* phosphorylation seem to be identical.

Antiserum was raised to the purified phosphoproteins secreted by B77 Rat 1 cells. $^{32}\text{PO}_4$ -labelled conditioned media from several cell lines were immunoprecipitated with this antiserum. Figure 1 shows that the transformation-dependent phosphoproteins derived from the various species are, in fact, all antigenically related. The size and degree of heterogeneity of the phosphoproteins vary (Fig. 1) and seem to be dependent on the species from which the transformed cell line was derived rather than on the transforming agent¹. There are several possible explanations for the apparent size differences and heterogeneity. The various phosphoproteins may have identical amino acid sequences but differ in degree of phosphorylation or proteolytic processing. Alternatively, the phosphoproteins may have similar but not identical primary structure. In any event, all show transformation sensitivity, and rat phosphoprotein antigens are present among the phosphoproteins of the other species.

In order to establish that the transformation-specific phosphoproteins are synthesized by the cells and do not arise by phosphorylation of serum proteins, the antiserum raised against the ts B77 Rat 1 (34°C) phosphoproteins was preabsorbed with fetal calf serum (in which cells were cultured). This preabsorption did not alter the extent or specificity of the immunoprecipitation of the phosphoproteins of MW 62,000 (62K) (not shown). In addition, when the ts B77 Rat 1 cells (34°C) were cultured and labelled with $^{32}\text{PO}_4$ in the absence of serum, the same amounts of 62K phosphoproteins were found in the conditioned medium (not shown). These results indicate that the 62 K phosphoproteins

The relevance of the three factors to mammalian melanogenesis remains to be demonstrated. There are, however, numerous reports of skin and coat colour regulation which might be explained by the actions of one or more of these factors. Three examples are tyrosinase-positive albinism in humans¹⁶, the annual pelage colour cycle in the Siberian hamster⁵, and in general the wide variation in mammalian coat colours¹. Also, the MSH-mediated sensitization of melanoma cells to dopachrome shown in Fig. 4 is likely to be explained by the factors we have described and is of relevance to the establishment of a rational therapy for human melanoma. The first step towards a full understanding of the physiological role of these factors is the elucidation of their molecular structures, a goal that we are now pursuing.

We thank Karen Vane and Marilyn Murray for providing cells, Dr Aaron B. Lerner for 5,6-dihydroxyindole, and Drs Saul Lande and Gisela Moellmann for helpful discussions. Supported by grants from the USPHS and The American Cancer Society.

Received 5 February; accepted 5 June 1980.

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Present addresses: *Department of Pathology, Beth Israel Hospital, Boston, Massachusetts 02215. †Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138.

are not fetal calf serum proteins that are simply phosphorylated by the cells.

To obtain further evidence that the phosphoproteins are synthesized by the cell, ts B77 Rat 1 cells (34°C, transformed phenotype) were preincubated in the presence and absence of cycloheximide and then labelled with $^{32}\text{P}\text{O}_4$ in the continued presence or absence of the drug. As shown in Fig. 2, cycloheximide treatment abolishes secretion of the 62K phosphoproteins, indicating that protein synthesis is required for their phosphorylation and release from the cells.

The cell-associated forms of the B77 Rat 1 phosphoproteins were identified by immune precipitation of ^{32}P -labelled cellular lysates. Figure 3 shows the results of such an experiment, which also shows the specificity of the antiserum. From among the very large number of phosphoproteins present in cell lysates (Fig. 3, track g) the antiserum bound only three proteins, all of MW about 62,000 (Fig. 3, track d). These intracellular 62K proteins have mobilities identical with those found in the conditioned medium after a chase (Fig. 3, track f). We were unable to chase completely the radioactivity in

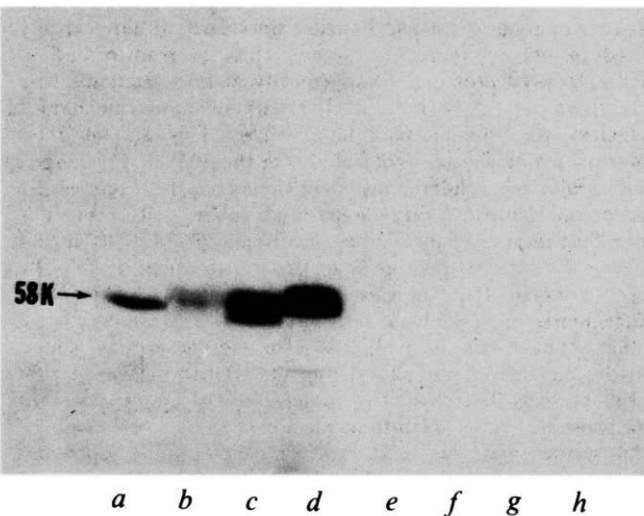


Fig. 1 ^{32}P -labelled conditioned media from various transformed cell lines were immunoprecipitated with antibody to the 62K transformation-specific secreted proteins from B77 Rat 1 cells. Cells and culture conditions, metabolic labelling with ^{32}P -orthophosphate, immunoprecipitation, SDS-polyacrylamide (7.5%) gel electrophoresis and autoradiography were as previously described¹. Antiserum was prepared as follows. ts B77 Rat cells were grown to confluence at 34°C in roller bottles, washed three times with serum-free medium and incubated overnight with serum-free medium (200 ml per roller bottle). Conditioned medium (1,800 ml) was spun at 10,000 r.p.m. for 10 min and passed over a water-washed DE-11 cellulose (Whatman) column (100 ml). The DE-11 cellulose had been pre-swollen in acid and base as recommended by Whatman and washed extensively with distilled water before loading. Virtually 100% of the transformation-dependent 62K phosphoprotein in the conditioned medium bound to the column (there actually seem to be several transformation-dependent phosphoproteins all with similar mobility corresponding to a MW of 62,000; see track d above and track f of Fig. 3). The loaded column was then washed with 1,000 ml of 0.25 M NaCl; this step removed contaminants without eluting the 62K phosphoproteins. The 62K phosphoproteins were eluted from the column with 0.5 M NaCl and concentrated by vacuum dialysis against phosphate-buffered saline. This antigen preparation was analysed by SDS-polyacrylamide gel electrophoresis, and it was determined that 90% of the protein had mobility identical to that of the 62K phosphoproteins. The remainder consisted of several other species in very low concentration. The 58K–62K transformation-dependent, non-phosphorylated proteins, which we have described previously¹, were not present in the antigen preparation because they do not bind to DE-11 at the ionic strength used here (our unpublished data). The rabbit was injected the first time (at eight dorsal sites) with 1 mg of 62K phosphoprotein in complete Freund's adjuvant. Three weeks later the injections were repeated with 1 mg protein and incomplete Freund's adjuvant. Antibody was present in serum 2 weeks later. The specificity of the antiserum is indicated in Fig. 3. a, Immune serum, NIL-8-HSV (hamster); b, immune serum, py3T3 (mouse); c, immune serum, Moloney MSV-MuLV SIRC (rabbit); d, immune serum, ts B77 Rat 1 (34°C) (rat); e, h, preimmune serum, media as in a, d.

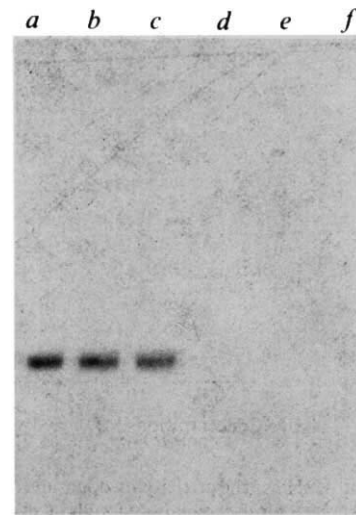


Fig. 2 Protein synthesis is required for secretion of the transformation-specific 62K phosphoproteins by ts B77 Rat 1 (34°C) cells. a, c, Media from control cells labelled for 1 h with ^{32}P -orthophosphate; d, f, media from cells preincubated with cycloheximide ($20\mu\text{g ml}^{-1}$) for 1 h before labelling for 1 h. The prominent band in tracks a, c corresponds to a MW of 62,000.

the 62K proteins from cell lysates, presumably because of the difficulty encountered in chasing phosphate pools². However, proportional amounts of pulse lysate and chase medium were found to contain identical quantities of 62K proteins (compare Fig. 3, tracks d and f), suggesting efficient secretion of the phosphoproteins synthesized during the pulse. Furthermore, the ratios of the different phosphate-labelled bands are the same in the pulse-labelled cells and the chase medium. These results indicate that all the 62K phosphoproteins are phosphorylated intracellularly and subsequently secreted into the culture medium in parallel.

The phosphoproteins secreted specifically by transformed cells can be phosphorylated *in vitro* by incubating the conditioned media with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The *in vitro* labelled phosphoproteins are precipitable with the antiserum which recognizes the *in vivo* labelled form (not shown). Furthermore,

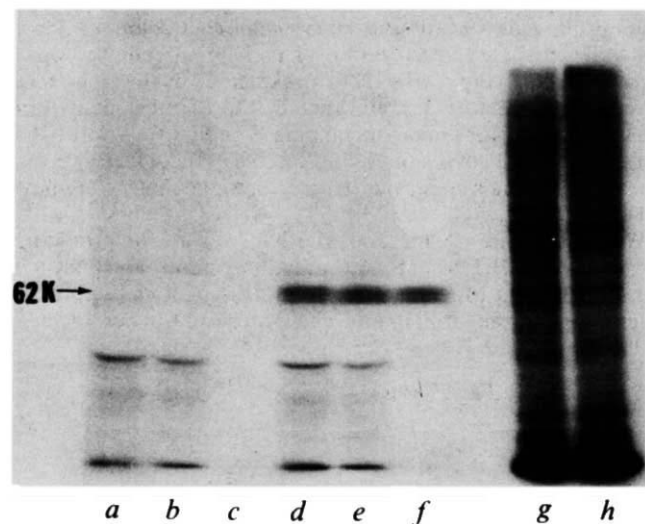


Fig. 3 ^{32}P -orthophosphate-labelled ts B77 Rat 1 (34°C, transformed phenotype) cell lysates and chase medium were immunoprecipitated with antibody to the 62K transformation-specific phosphoproteins. a, Preimmune serum, cell lysate, 45 min label; b, preimmune serum, cell lysate, 45 min label, 45 min chase; c, preimmune serum, equivalent amount of chase medium; d, immune serum, cell lysate, 45 min label; e, immune serum, cell lysate, 45 min label, 45 min chase; f, immune serum, equivalent amount of chase medium; g, total cell lysate, 45 min label, 1/10th the volume used in d; h, total cell lysate, 45 min label, 45 min chase, 1/10th the volume used in e.

a comparison of the phosphopeptides generated by partial proteolysis of the *in vitro* and *in vivo* labelled 62K phosphoproteins indicates that the major *in vitro* and *in vivo* sites of phosphorylation are likely to be identical (see Fig. 4).

Identical levels of *in vitro* phosphorylation of the 62K phosphoproteins (ts B77 Rat 1, 34 °C) were observed in serum-free conditioned medium and in conditioned medium containing 5% fetal calf serum (not shown). This result indicates that the protein kinase activity is secreted by the cells and is not a serum protein. We also found that conditioned medium from ts B77 Rat 1 cells (34 °C) can phosphorylate histones in the presence of ^{32}P -ATP, whereas unconditioned medium does not have this phosphorylating activity, again indicating secretion of protein kinase by the cells (data not shown). We have found that the phosphorylating activity secreted by B77 Rat 1 cells and ts B77 Rat 1 cells can be equally inactivated by preincubation at 41 °C. This suggests that the phosphorylating activity does not contain the lesion responsible for the temperature-sensitive phenotype of the ts B77 Rat 1 cells.

Experiments with serum from tumour-bearing animals^{3,4} have suggested that the transformation-specific phosphoproteins described here are antigenically distinct from pp60^{src}, the 60,000-MW phosphoprotein encoded by the *src* gene of avian sarcoma virus. Serum active against the endogenous *sarc* gene protein of both avian and mammalian cells⁵ also failed to precipitate the secreted phosphoproteins.

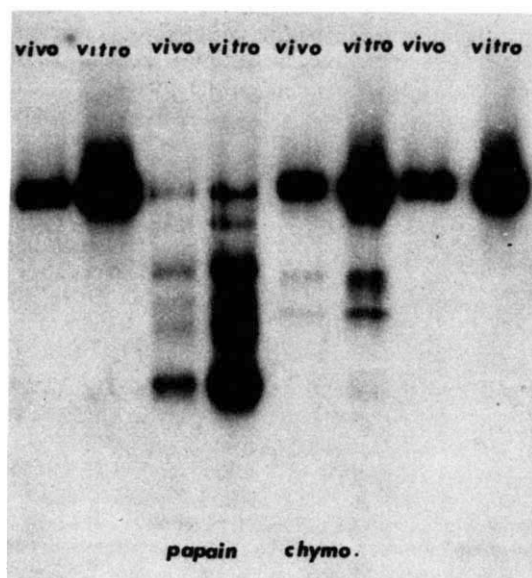


Fig. 4 Partial proteolysis of the 62K phosphoproteins from ts B77 Rat 1 (34 °C) cells labelled *in vivo* with ^{32}P -orthophosphate or *in vitro* with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Total labelled conditioned medium, labelled *in vivo* or *in vitro* as previously described¹, was electrophoresed on a 7.5% preparative slab gel. The 62K phosphoprotein band containing all the 62K species was cut from the dried preparative gel, rehydrated in Laemmli stacking gel buffer⁸ and electrophoresed on a 12.5% Laemmli gel with 250 µg papain or 50 µg chymotrypsin (chymo.), or no additions. 'vivo' 62K ^{32}P -labelled proteins from the media of cells labelled *in vivo* with $^{32}\text{PO}_4$; 'vitro', 62K proteins from the media of unlabelled cells, 62K proteins subsequently labelled *in vitro* with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

In addition, the transformation-specific phosphoproteins are unable to phosphorylate IgG in immune precipitates, an activity which is characteristic of pp60^{src} and pp60^{src} (refs 4–7). However, incubations of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and washed 62K phosphoprotein immunoprecipitates did result in ^{32}P labelling of the 62K phosphoprotein itself. Whether this represents autophosphorylation or phosphorylation by a separate kinase activity in the immunoprecipitate is not known.

To summarize, the phosphoproteins described here are closely correlated with the transformed phenotype. In cells which are temperature-sensitive for transformation, secretion

of the phosphoproteins is temperature-dependent. The phosphoproteins are synthesized by the cells and phosphorylated intracellularly and are not derived by phosphorylation of serum proteins. These transformation-dependent phosphoproteins are common to all mammals for which we have so far obtained transformed cell lines—hamster, rat, mouse and rabbit. Because of their antigenic relatedness, it seems likely that the transformation-specific phosphoproteins secreted by the various cell lines have a common function. Their close correlation with transformation suggests that they may be involved in the maintenance of some aspect of the transformed state.

We thank Carl Bryant and Antonia Destree for technical assistance, Naomi Foster for photography, Dr P. W. Robbins for his interest and helpful suggestions, and all those who provided the cells and antisera used in these studies. This work was supported by grants from the NIH, NCI (RO1 CA 17007 to R.O.H. and PO1 CA 14051 to S.E. Luria) and from the American Cancer Society (VC 258). R.O.H. is the recipient of an NCI Research Career Development Award.

Received 11 February; accepted 24 April 1980.

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Evidence that a developmentally regulated glycoprotein is target of adhesion-blocking Fab in reaggregating *Dictyostelium*

Claudia Steinemann & Roger W. Parish

Department of Cytology, Plant Biology Institute, University of Zürich, Zollikerstrasse 107, CH 8008 Zürich, Switzerland

The target site of adhesion-blocking Fab in aggregating *Dictyostelium discoideum* cells is a plasma membrane glycoprotein of approximate molecular weight 82,000 (refs 1–3), which is no longer synthesized after aggregation, and disappears from the plasma membrane^{4,5}. However, since cell adhesion remains important during later stages of differentiation^{6–8}, presumably another protein(s) takes over its function. One candidate is a glycoprotein (molecular weight 95,000) whose synthesis commences at the tip stage and continues until the completion of development^{5,8}. Both proteins are strongly antigenic⁴. When pseudoplasmodia ('slugs', a late developmental stage) are disaggregated and replated, the cells recapitulate the previous sequence of morphological changes much more quickly than before^{6–8}, although the glycoprotein of molecular weight 82,000 is not resynthesized during reaggregation^{4,8}. We report here that monovalent antibody (Fab) directed against slug plasma membranes inhibits reaggregation by binding to the glycoprotein of molecular weight 95,000 which thus seems to be involved in cell adhesion.

Antisera against slug plasma membranes contained antibodies specific for a number of proteins (Fig. 1a). Apart from actin, the major antigen in the membrane was a glycoprotein of molecular weight 95,000 (ref. 4, Fig. 1a). This antigen could be extracted from the plasma membrane with butanol and was the major antigen in the butanol extract (Fig. 1b). Radioactive

acetate and glucosamine were incorporated into this glycoprotein from the tip stage until late culmination⁵ (Fig. 1c, d). The glycoprotein was not present in the plasma membrane of amoebae or aggregating cells^{4,5}.

When slug cells were disaggregated and the single cells shaken in buffer, aggregates had already formed after 45 min (Fig. 2a). Reaggregation was inhibited when Fab directed against slug plasma membrane was present (Fig. 2b). Fab directed against plasma membranes from vegetative or early aggregation cells had no effect on reaggregation (Fig. 2d). When a butanol extract from slug plasma membrane was incubated with the anti-slug Fab, reaggregation was no longer inhibited (Fig. 2c). Hence, antigens in the butanol extract were evidently competing with cells for adhesion-blocking Fab.

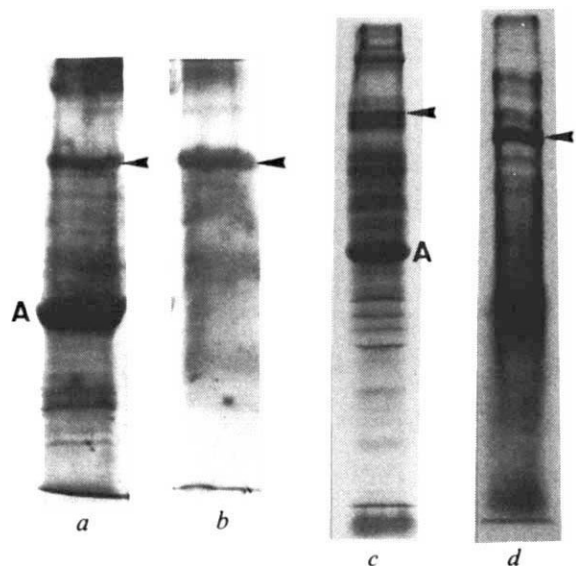


Fig. 1 The plasma membrane of slug cells: antigens and proteins incorporating L-[1-¹⁴C]fucose and [1-¹⁴C]acetate. Growth of *D. discoideum* NC-4 (wild type) cells, labelling of cultures with isotopes, plasma membrane isolation, SDS-polyacrylamide gel electrophoresis, autoradiography, preparation of antisera and detection of antigens on gels were performed as described previously^{4,5,9}. Plasma membranes were injected into rabbits in Freund's Complete Adjuvant; injection in saline resulted in a low titre of antibody directed against the antigen of molecular weight 95,000, (indicated by the arrow). *a*, Antigens in total slug plasma membrane; SDS-polyacrylamide gels were incubated with antisera raised against slug plasma membranes. (A = actin; molecular weight 42,000.) *b*, Antigens in the butanol extract of slug plasma membranes; SDS-polyacrylamide gels incubated with antisera as in *a*. *c*, Autoradiograph of SDS-polyacrylamide gel of slug plasma membrane after labelling from the tip stage with [1-¹⁴C]acetate. *d*, Autoradiograph of plasma membrane from early slug stage after labelling from mid-aggregation with L-[1-¹⁴C]fucose.

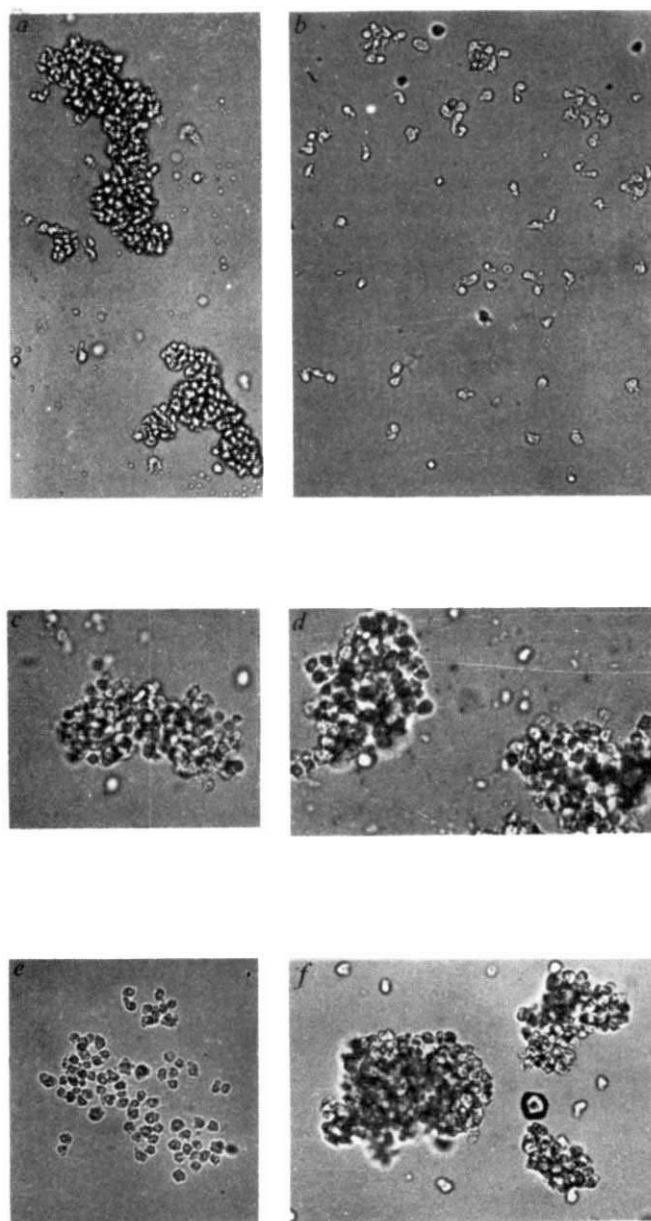


Fig. 2 The effects of Fab on the reaggregation of disaggregated slug cells. Fab was purified as described previously⁹. Reaggregation was assayed by incubating disaggregated slug cells (2×10^7 cells ml⁻¹) in a roller culture (40 r.p.m., Multipurpose Rotator, Scientific Industries) in 20 mM KCl, 24 mM MgCl₂ and 10 mM EDTA in 0.05 M Sørensen's phosphate buffer (pH 6.5). Fab was added to give a final concentration of 1 mg protein ml⁻¹. Removal of Fab specific for the antigen of molecular weight 95,000 was accomplished in the following way. Plasma membranes were isolated from vegetative cells (3.6×10^9) and extracted with butanol. The extract (1.1 mg protein in 370 μ l) was electrophoresed on SDS slab gels (18 lanes). The region corresponding to the antigen of molecular weight 95,000 was cut out and SDS removed by shaking the gel pieces in 25% isopropanol/10% acetic acid solution for 20 h, changing the solution once. The gel pieces were then washed with 15 mM Na phosphate buffer (pH 6.8) for 3 h with one change of buffer. The slices were shaken for 6 h with 200 μ g Fab in 2.0 ml PDF solution (20 mM KCl, 25 mM MgCl₂ in 0.05 M Sørensen's phosphate buffer, pH 6.5). The PDF solution containing Fab which had not bound to the antigen in the gel was removed and the gel shaken overnight in 2.0 ml PDF solution to further remove any non-specifically bound Fab. The two PDF solutions were combined (4.0 ml) and concentrated by vacuum dialysis (final volume 0.20 ml). Hence, a Fab fraction was obtained from which the Fab binding to the antigen of molecular weight 95,000 had been at least partially removed. As controls, Fab was incubated with other regions of the gel. Cells were photographed after 45 min in the roller culture. *a*, Control; *b*, anti-slug Fab; *c*, anti-slug Fab plus proteins extracted from slug plasma membranes using butanol; *d*, Fab (against aggregation competent cells); *e*, anti-slug Fab remaining after incubation with a gel slice from the region of the gel corresponding to molecular weights 80,000–90,000; *f*, anti-slug Fab after removal of Fab specifically binding to the antigen of molecular weight 95,000.

We determined whether the antigen of molecular weight 95,000 was involved in cell adhesion by cutting the antigen from SDS gels and using the gel slices to remove the specific Fab from the total Fab⁹. The remaining Fab (more than 98% of total Fab) was subsequently unable to inhibit reaggregation (Fig. 2f). Preincubation of anti-slug Fab with another region of the gel (such as the region representing molecular weights 80,000–90,000) did not prevent Fab inhibiting reaggregation (Fig. 2e).

Anti-slug Fab was also incubated with aggregation-competent cells to remove any Fab directed against contact sites for the protein of molecular weight 82,000 that might be present. The remaining Fab still inhibited reaggregation of disaggregated slug cells. Hence the results were not due to contamination with Fab against this antigen. The anti-slug Fab did not block the EDTA-resistant adhesion of aggregation-competent cells or the EDTA-sensitive adhesion of vegetative

cells. (Fab against aggregation-competent cells did, however, block the adhesion of these cells.)

Recently, Geltosky *et al.*¹⁰ described a glycoprotein of molecular weight 150,000, present on the surface of vegetative *D. discoideum* cells, whose concentration increased during the first 10 h of development. Fab directed against this protein blocked the ability of aggregation-competent cells to reaggregate. However, the synthesis of such a glycoprotein was not detected in labelling experiments⁵. (A glycoprotein of molecular weight 155,000 was synthesized later, between early aggregation and culmination⁵.) One explanation for this discrepancy would be that the glycoprotein described by Geltosky *et al.*¹⁰ is stored in the cytoplasm of vegetative cells and transported to the cell surface during early development. It may bind to one of the glycoproteins of molecular weights 82,000 or 95,000 on adjacent cells. In our plasma membrane preparations no proteins with molecular weights of approximately 150,000 were markedly antigenic. Hence, our Fab contained few, if any, molecules directed against the glycoprotein of Geltosky *et al.*¹⁰. When the region of the gel corresponding to a molecular weight of 150,000 was preincubated with anti-slug Fab, the remaining Fab still inhibited aggregation.

Our results suggest that the antigen of molecular weight 95,000 is involved in cell adhesion, although we cannot be absolutely sure that it is not another protein running at around that region on gels that is responsible. However, we consider this unlikely as butanol extracts contained no other detectable proteins which ran so near to this antigen as to be included in the gel slice. The possibility that binding of Fab to the antigen of molecular weight 95,000 indirectly inhibits reaggregation by selective steric impairment of another set of molecules cannot be excluded.

The plasma membrane glycoproteins involved in cell adhesion during the early developmental stages of both *D. discoideum* (molecular weight 82,000)² and *Polysphondylium pallidum*¹¹ (molecular weight 71,000)⁹ are no longer synthesized when aggregation is complete and are subsequently lost from the cells^{4,5,8,9}. This may be related to the sorting out of pre-spore and pre-stalk cells that occurs during the post-aggregation phase¹²⁻¹⁶. The glycoprotein of molecular weight 95,000 of *D. discoideum*, for example, may be restricted to one cell type or occur at different concentrations on the two types. We are using immunofluorescence to check these possibilities. The glycoprotein of molecular weight 150,000 apparently does not disappear from the plasma membrane during differentiation¹⁰. However, it may be part of a second type of adhesion which is not involved in sorting out. Alternatively, a change in only one component of an adhesion system may be sufficient to induce sorting out.

We thank Dr C. R. Parish for providing the antisera. This work was supported by the Schweizerische Nationalfonds zur Förderung der wissenschaftlichen Forschung (grant no. 3.421-0.78).

Received 18 February; accepted 5 June 1980.

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Modulation of brain polyphosphoinositide metabolism by ACTH-sensitive protein phosphorylation

J. Jolles, H. Zwiers, C. J. van Dongen, P. Schotman, K. W. A. Wirtz* & W. H. Gispen

Division of Molecular Neurobiology, Rudolf Magnus Institute for Pharmacology and Laboratory for Physiological Chemistry, Medical Faculty, Institute of Molecular Biology, State University of Utrecht
*Laboratory of Biochemistry, State University of Utrecht, Padualaan 8, Utrecht, The Netherlands

Phosphorylation of membrane components is thought to be an important process in membrane function¹. Phosphorylated proteins² and a special class of phospholipids, the (poly)phosphoinositides (poly PI)³, are implicated in the regulation of membrane permeability and synaptic transmission in neurones. For many years, protein phosphorylation and poly PI metabolism have been studied in parallel without knowledge of their possible interaction. We report here that the ACTH-sensitive protein kinase/B-50 protein complex which we recently isolated in soluble form from rat brain synaptosomal plasma membranes^{4,5} has lipid phosphorylating activity. Exogenously added phosphatidylinositol 4-phosphate (DPI) is phosphorylated to phosphatidylinositol 4,5-diphosphate (TPI), and this DPI-kinase activity is dependent on the state of phosphorylation of the protein kinase/B-50 protein complex. The results imply that phosphorylation of protein may affect the metabolism of (poly)PI in brain cell membranes.

Effects of peptide hormones on phosphatidylinositol (PI) metabolism in cell membranes of peripheral tissues have been obtained⁶⁻⁸. Similarly, corticotropin (ACTH) is known to affect (poly)PI metabolism in membrane fractions from rat brain⁹. An enhanced metabolism of TPI was also found in response to cholinergic and noradrenergic receptor stimulation¹⁰ and after the influx of Ca²⁺ into the cell¹¹. With respect to the phosphorylation of proteins, we found that ACTH exerts a dose- and structure-dependent inhibitory effect on the *in vitro* phosphorylation of specific proteins in synaptosomal plasma membranes⁴. The ACTH-sensitive protein kinase (molecular weight 71,000; isoelectric point 5.5) and one of its substrate proteins (B-50; molecular weight 48,000; isoelectric point 4.5) were isolated and the phosphorylation of the B-50 protein was found to be Ca²⁺-dependent⁵.

The protein kinase/B-50 complex used in the present study was obtained from a Triton-KCl solubilized synaptosomal plasma membrane fraction and subjected to further purification by DEAE-cellulose chromatography and ammonium sulphate precipitation (ASP) as described before (see legend to Fig. 1). The ASP fraction has been characterized by SDS-polyacrylamide slab gel electrophoresis: the position of B-50 and its protein kinase are indicated in Fig. 1a. Upon incubation of this ASP fraction with [γ -³²P]ATP only the B-50 protein became labelled (Fig. 1b). Interestingly, addition of DPI to the ASP fraction in the phosphorylation medium produced [³²P]TPI as shown by autoradiography after thin layer chromatography of the lipid extract (Fig. 1c).

When the eluate of the DEAE-cellulose column was assayed for B-50 protein and DPI-kinase activity, it was found that the two activities cochromatographed, suggesting a possible relationship (J.J. *et al.*, in preparation). Confirming previous results⁶, incubation of the ASP fraction in the presence of ACTH₁₋₂₄ resulted in a dose-dependent inhibition of B-50 phosphorylation (Table 1). Concomitantly, the phosphorylation

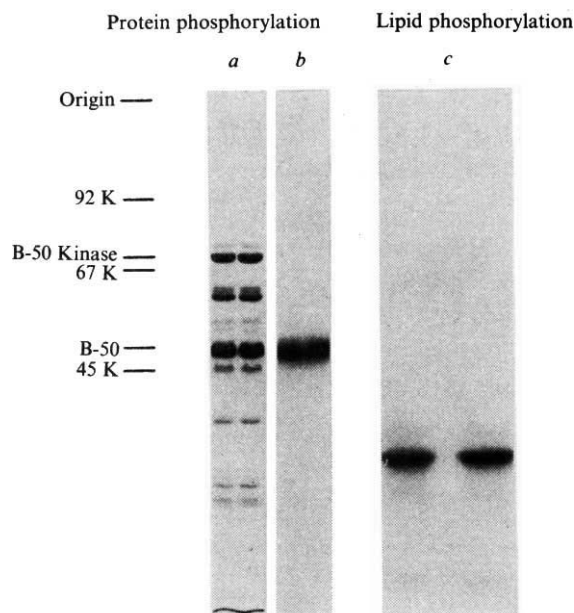


Fig. 1 *a*, Gel staining pattern showing the position of the protein bands, and *b*, autoradiogram showing the endogenous phosphorylation profile of the ASP₅₅₋₈₀ fraction. Each track contains 6 μ g of total ASP protein. The position of molecular weight marker proteins, and of B-50 and B-50 kinase is indicated. *c*, Autoradiogram after extraction and thin layer chromatography of ASP fraction incubated with exogenous DPI. The position of DPI and TPI is indicated. Each lane shows the results of duplicate determinations. The ACTH-sensitive protein kinase and its protein substrate, the B-50 protein, were isolated as described by Zwiers *et al.*^{5,6}. Briefly, a crude synaptosomal plasma membrane fraction was prepared from rat brain. The membrane-bound B-50 protein kinase activity was solubilized with 0.5% Triton X-100 with 75 mM KCl in 6 mM Tris, 0.1 mM dithiothreitol (pH 8.1) and the solubilized proteins separated by DEAE-cellulose chromatography. The proteins were eluted with a salt gradient in 10 mM Tris, 1 mM CaCl₂, 0.1 mM dithiothreitol, pH 7.4 (buffer A). The eluate fractions containing the peak of B-50 protein and B-50 phosphorylating activity were pooled and subjected to 55% ammonium sulphate saturation. After separation of the precipitated proteins by centrifugation the supernatant was saturated to 80% with ammonium sulphate and the precipitate collected by centrifugation. This ASP₅₅₋₈₀ fraction was dissolved in 400 μ l buffer A and dialysed overnight against 1 l of buffer A. The phosphorylation assay was performed in the following conditions: 50 mM Na acetate, 10 mM Mg acetate, 1 mM Ca acetate (pH 6.5), 7.5 μ M ATP, 2 μ Ci [γ -³²P]ATP (~3,000 Ci mmol⁻¹, Amersham), 7.5 μ l ASP fraction, 40 μ M DPI (disodium salt, Sigma) in a final volume of 25 μ l. The enzyme fraction was routinely preincubated for 5 min before the incubation was started by the addition of DPI and ATP. Buffer with or without ACTH₁₋₂₄ was added 15 s before the ATP and incubation continued for 15 min unless indicated otherwise. Protein phosphorylation and lipid phosphorylation assays were always run in parallel. The protein phosphorylation reaction was terminated by the addition of a denaturing solution and the proteins were separated by SDS-polyacrylamide gel electrophoresis. After staining with Fast Green FCF and autoradiography the labelled protein bands were cut from the gel and measured for radioactivity by liquid scintillation counting^{4,5}. The lipid phosphorylation reaction was terminated by the addition of ice-cold chloroform/methanol/13 N HCl solution (200:100:0.75 v/v). Carrier poly PI were added and an acid extraction was carried out. The lipid extract was separated by one-dimensional high performance thin layer chromatography (HPTLC) on precoated HPTLC plates (Merck) impregnated with 1% K oxalate. After lipid staining and autoradiography the labelled lipids were scraped from the plate and counted for radioactivity in a liquid scintillation counter^{8,12}. All experiments were performed at least three times.

of exogenously added DPI to TPI was stimulated (+35% and +149% at 10 μ M and 100 μ M ACTH respectively). Because similar ACTH effects have been obtained with brain synaptosomal membrane fractions^{4,12}, it seems very probable that the enzymes responsive to the peptide in synaptosomal plasma membranes are present in the ASP fraction. The correlation between inhibited B-50 phosphorylation and stimulated TPI formation suggests that the state of B-50 phosphorylation may affect the DPI kinase activity (see ref. 13 for a recent review on the regulation of enzymatic activity by phosphorylation). To test this hypothesis the ASP fraction was preincubated with [γ -³²P]ATP for various periods of time, and DPI kinase activity was

measured after this B-50 prephosphorylation period. As Fig. 2*a* shows, the extent of B-50 protein phosphorylation increased with time up to 40 min but DPI kinase activity diminished with increasing B-50 phosphorylation, providing additional evidence for a direct relationship between B-50 protein and DPI phosphorylation. It appears that the formation of TPI is especially affected by the final 30% of the protein phosphorylation; more insight into the exact molecular mechanisms involved is needed to appreciate the significance of this finding more fully. To eliminate the possibility that the decreased formation of TPI is due to enzyme breakdown during the phosphorylation period,

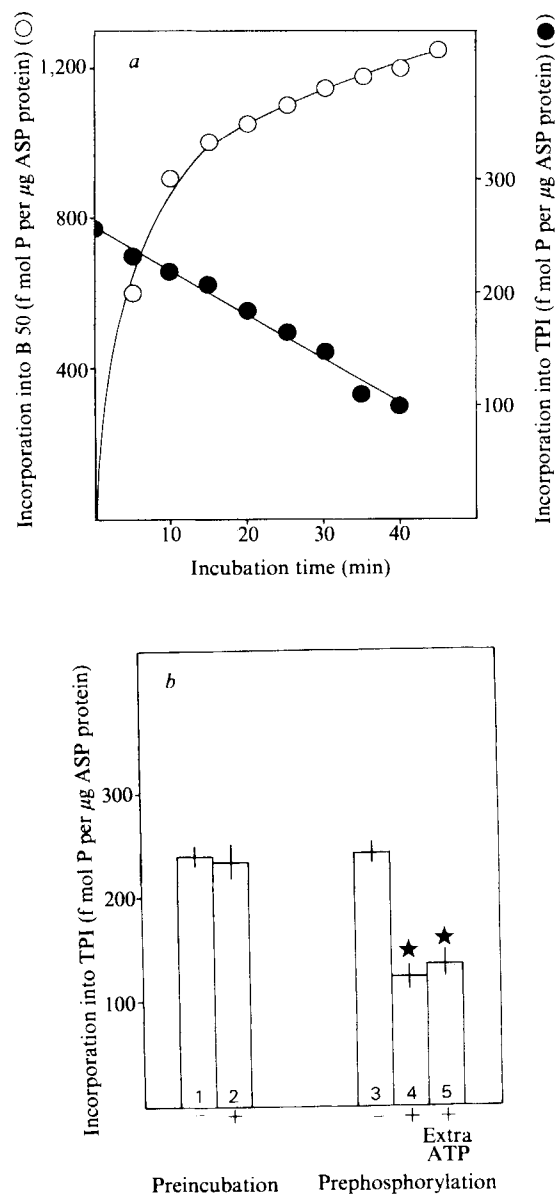


Fig. 2 *a*, The effect of B-50 prephosphorylation on TPI formation. Incubations were performed as described in the legend to Fig. 1, with the following modifications: ASP fractions were incubated in the absence of DPI for 5–45 min (○). Exogenous DPI was added to the ASP fraction after the various prephosphorylation time periods (0–40 min) and incubation continued for another 5 min. The amount of TPI formation in these 5 min periods (0–5, 5–10 min etc.) was measured (●). *b*, The effect of B-50 prephosphorylation on TPI formation: influence of preincubation and ATP. ASP fractions were incubated for 15 min in the presence of ATP and DPI as indicated in the legend to Fig. 1 (group 1 and 3). Group 2 was preincubated for 30 min before addition of DPI and ATP. Group 4 was preincubated for 30 min in the presence of ATP before DPI was added and the incubation continued for another 15 min. Group 5 was treated like group 4 but extra ATP containing 2 μ Ci [γ -³²P]ATP was added together with the DPI to give a final concentration of 15 μ M ATP.

TPI formation was measured with and without 30 min preincubation in the absence of ATP. As Fig. 2b shows, preincubation had no effect. Furthermore, the decreased TPI formation seemed not to be related to exhaustion of ATP during the prephosphorylation, as the amount of labelled lipid was significantly depressed even after the addition of extra ATP (Fig. 2b). These results are in line with those obtained with ACTH (Table 1 and refs 4, 5, 12): there seems to be an inverse relationship between B-50 phosphorylation and TPI formation. Therefore, and in view of the studies on enzyme phosphorylation¹³, we propose that the effect of ACTH on TPI production is secondary to the effect on protein phosphorylation: the peptide inhibits the B-50 kinase⁴, and the B-50 substrate protein in its non-phosphorylated form, stimulates TPI formation. It awaits further experimentation to determine whether the B-50 protein is a regulatory factor of the DPI-kinase or the kinase itself.

Table 1 The effect of ACTH₁₋₂₄ on B-50 phosphorylation and TPI formation

Treatment	Incorporation (f mol P per µg ASP protein)		Increase	
	B-50		(%)	(%)
No addition	994.9±8.0			
ACTH ₁₋₂₄ (10 µM)	517.3±21.2		-48†	+35*
ACTH ₁₋₂₄ (100 µM)	308.4±25.4		-69†	+149†

Incubations were performed as described in the legend to Fig. 1. Values represent mean ± s.e.m. (n = 5). Differences were tested with Student's t-test.

* P < 0.01.

† P < 0.001.

Interestingly, Takai *et al.*^{14,15} recently demonstrated that the activity of a Ca²⁺-dependent protein kinase is linked to PI and its breakdown product 1,2-diacylglycerol, suggesting that a functional relation exists between protein phosphorylation and phosphatidylinositol metabolism. DPI and especially TPI are very potent chelators of Ca²⁺ and Mg²⁺ (refs 3, 16); they interact strongly with proteins and, due to their very negative headgroup which contains three (DPI) or five (TPI) negative charges, these lipids may carry the negative potential of the membrane^{17,18}. Thus a change in the relative amounts of the phosphoinositides PI/DPI/TPI in the membrane may affect the conformation of membrane proteins and change the amount of Ca²⁺ and Mg²⁺ bound to the membrane. Both protein phosphorylation^{1,2} and (poly)PI metabolism³ are implicated in the regulation of membrane permeability and synaptic transmission in neurones. The results obtained in the present study suggest that the phosphorylation of a quantitatively minor membrane protein (B-50 protein), may bring about profound changes in membrane characteristics by changing the content of poly PIs. The demonstration that ACTH influences this process may suggest new avenues for investigating the biochemical mechanisms underlying agonist-effector cell membrane interactions.

Received 14 March; accepted 27 May 1980.

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Chemically induced myotonia in amphibia

A. H. Bretag, S. R. Dawe & A. G. Moskwa

South Australian Institute of Technology, North Terrace, Adelaide, South Australia, Australia

Frogs and toads treated with high doses of anthracene-9-carboxylic acid (A-9-C) develop prolonged muscular contractions and 'divebomber' electromyograms characteristic of myotonia. Hitherto, myotonia has been considered peculiar to homeotherms where it is associated with several hereditary diseases and can be induced by specific treatments, most of which seem to act by decreasing membrane chloride conductance¹. Our work indicates that myotonia can be induced in amphibia by similar means. We offer possible reasons why others have missed seeing myotonia in amphibia.

Toads, *Bufo marinus*, weighing about 120 g and frogs, *Littoria aurea*, of 30-60 g were kept at room temperatures of 20-25 °C and injected subcutaneously with A-9-C (167 mg per kg as a 10 mg ml⁻¹ solution neutralized with NaOH). Myotonic behaviour could then be observed in the toads within half an hour. In the frogs it took a number of hours to develop. If jumping was provoked it resulted in one or a few normal jumps followed by jumps with severe extensor spasm on landing. This often caused the animal to freeze in a bizarre attitude for some seconds (Fig. 1), before slowly relaxing and then resuming a normal squatting position. Continued provocation to jump eventually resulted in a return to normal. After rest for half an hour, however, the whole cycle could be repeated. This kind of response could be obtained for 2 or 3 days following the injection of A-9-C after which jumping behaviour gradually returned to normal over the next few days.

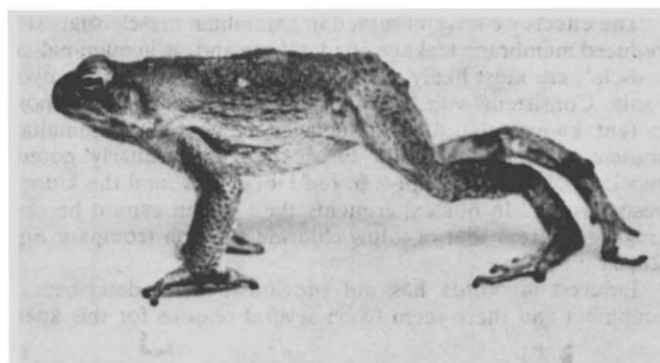


Fig. 1 An A-9-C treated toad, still immobile and stiff-legged, ~3s after landing from a jump. We have occasionally seen these animals overbalance on landing to become supported on their forelegs and snout with their hind legs extended above them. By contrast, untreated toads jump, land and resume their normal squatting position within half a second.

While animals were displaying myotonic behaviour, audio electromyograms could be recorded from leg muscles with frequent 'divebomber' bursts of activity in response to slight movements of the concentric recording needle. After exercise 'warm up', divebomber sounds were virtually eliminated.

Sartorius muscles, *in vitro*, could also be made to produce myotonic contractions (Fig. 2a) in response to A-9-C at concentrations comparable to those expected to pertain in the *in vivo* experiments. Similar results could be obtained using 4,

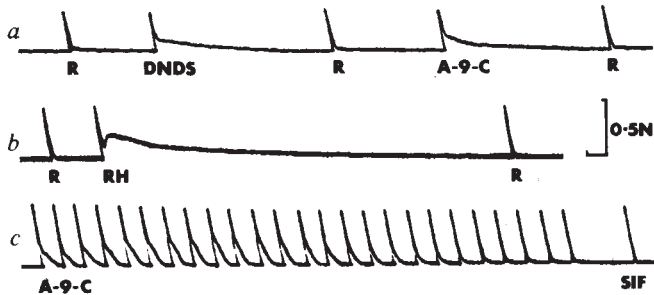


Fig. 2 Isometric tension records from isolated frog and toad sartorius muscles bathed at room temperature (20 °C) in Ringer solution² (R) and synthetic interstitial fluid³ (SIF), respectively. Muscles were allowed to equilibrate in particular solutions for 10 min before stimulation with a train of three, just supramaximal, 1 ms pulses⁴ at 225 Hz. *a*, Frog sartorius muscle in control solution (R) and with the addition of DNDS (2 mmol l⁻¹) and A-9-C (2.25 mmol l⁻¹) showing that normal relaxation is interrupted and prolonged by these agents. Time scale, 1 s. *b*, Frog sartorius muscle. Response in control solution (R) compared with response in a chloride-free solution (RH) made by substituting appropriate quantities of sodium 3,5-diacetamido-2,4,6-triiodobenzoate (Hypaque, Winthrop) for sodium chloride, and potassium and calcium sulphate for their chlorides. Cationic activities were maintained constant⁵. Time scale, 1 s. *c*, Toad sartorius muscle. Record of contractions resulting from stimulation at 5 s intervals in the presence of A-9-C (2.25 mmol l⁻¹). After about 20 such stimuli the prolonged relaxation returns to normal, as illustrated in the final contraction of the record which was initiated 10 min after washout of the A-9-C with SIF. Time scale, 5 s.

4'-dinitro-2, 2'-stilbenedisulphonic acid (DNDS, Fig. 2*a*), furosemide (not shown) or a chloride-free bathing solution (Fig. 2*b*). Muscles were stimulated only once every 10 min as 'warm up' was rapidly produced by more frequent stimuli (Fig. 2*c*). Typical myotonic after-discharges of action potentials could be found in A-9-C treated toad sartorius muscle fibres following constant current stimulation (Fig. 3). An increase in input resistance was observed in these A-9-C treated fibres.

The effects we have observed in amphibian muscle suggest a reduced membrane leakage conductance and, as in mammalian muscle¹, are most likely to be low chloride-conductance myotonia. Consistent with this interpretation, A-9-C is the most potent known chloride conductance blocker in mammalian muscle⁶ while DNDS and furosemide are similarly potent blockers of anion transport in red blood cells⁷ and the kidney respectively⁸. In our experiments their action cannot be distinguished from that of a low chloride solution (compare Fig. 2*a, b*).

Induced myotonia has not previously been described in amphibia and there seem to be several reasons for this apart

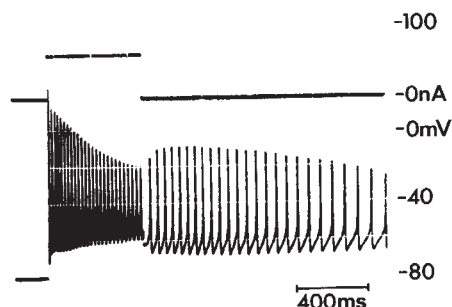


Fig. 3 Intracellular 3M KCl microelectrode recording of a myotonic after-discharge of action potentials in a single fibre of toad sartorius muscle bathed in SIF containing A-9-C (2.25 mmol l⁻¹) at 20 °C. The fibre was stimulated by the constant current pulse shown in the upper trace delivered through a second microelectrode (2M K citrate) inserted close to the voltage recording electrode.

from the possibility of species differences. First, the doses of A-9-C that we have used are considerably higher than those used by others⁹ and the long delay before significant behavioural effects appear in frogs may have caused slight myotonia to be overlooked. Second, since exercise quickly produces 'warm up', myotonia is best seen if the animals are closely confined for half an hour before testing.

We thank the Muscular Dystrophy Association of S.A. and the Research Committee of the South Australian Institute of Technology for support.

Received 3 March; accepted 2 June 1980.

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Control of tension development in scallop muscle fibres with foreign regulatory light chains

R. M. Simmons & A. G. Szent-Györgyi

Department of Physiology, University College London, Gower Street, London WC1E 6BT, UK, and Department of Biology, Brandeis University, Waltham, Massachusetts 02154

We have shown previously that chemically skinned fibre bundles from scallop muscle can be desensitized to the action of calcium by the removal of the regulatory light chains of myosin and that sensitivity can be restored by the re-addition of scallop light chains¹. We have now confirmed these results and extended our observations to fibre bundles from which one or both of the regulatory light chains per myosin have been removed (by treatment with EDTA at 7 and 25 °C, respectively) and replaced by the corresponding light chains from other species. In the case of a double substitution—where both light chains were replaced—sensitivity was restored completely by light chains from other molluscs and from gizzard muscle; for a single substitution there was restoration of sensitivity by all the light chains tested.

The tension response of a chemically skinned fibre bundle to an activation-relaxation sequence is shown in Fig. 1*a*. There was no tension development in relaxing solution ($[Ca^{2+}] < 10^{-10}$ M) and maximal development in activating solution ($[Ca^{2+}] = 10^{-4.8}$ M). Treatment with EDTA at 25 °C removed both regulatory light chains, as shown in other preparations by urea gel electrophoresis, and tension development was maximal in relaxing solution as well as in activating solution (Fig. 1*b*). Thus, the fibre bundle was no longer sensitive to calcium, in the sense that regulatory control had been completely lost.

Fully desensitized fibre bundles were exposed to light chains from the same and other species, tested for their sensitivity to calcium and analysed for light chain content by urea (and in some cases SDS) gel electrophoresis. The results are summarized in Table 1. In most cases, two light chains per myosin were successfully combined. Preparations treated with light chains from the scallop and other molluscan species (spisula, mercenaria and loligo) showed a complete restoration of sensitivity towards calcium (Fig. 1*c*). Sensitivity was also restored by the combination of chicken gizzard light chains (Fig. 1*d*), but rabbit skeletal, bovine cardiac and lobster tail muscle light chains had little or no effect (Fig. 1*e*). Some fibre bundles which

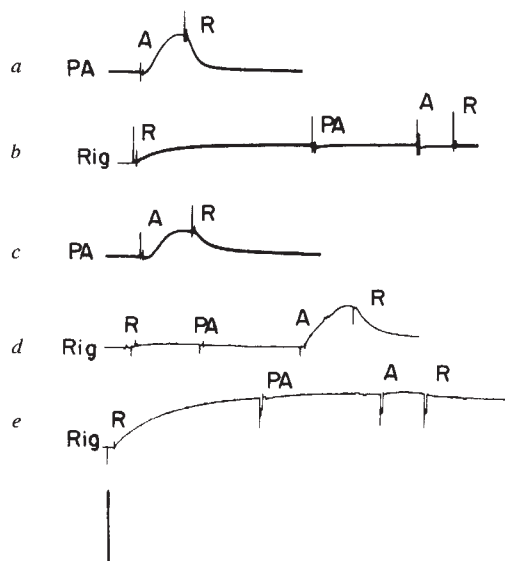


Fig. 1 Double-substitution experiments. Tension responses of skinned fibre bundles to activation-relaxation sequences; effects of complete removal of scallop regulatory light chains and of replacement by light chains from the scallop and other species. *a-c*, Same fibre bundle; *a*, before EDTA treatment; *b*, desensitized by treatment with EDTA at 25 °C (control); *c*, resensitized with scallop light chains; *d*, complete substitution of scallop light chains by gizzard light chains; *e*, complete substitution by lobster light chains. Fibre bundles were from the striated adductor muscle of *Placopecten magellanicus*. Removal and addition of light chains were as described in Table 1 legend. Symbols for solutions: PA, pre-activating (a relaxing solution with low [EGTA] to increase rate of tension development in activating solution); A, activating; R, relaxing; Rig, rigor. Solutions⁶ (concentrations in mM unless stated): PA; EGTA 0.1, HDTA 29.9, $[Ca^{2+}] \sim 10^{-8}$ M. A; CaEGTA 30, $[Ca^{2+}] = 10^{-4.8}$ M. R, Rig; EGTA 30, $[Ca^{2+}] < 10^{-10}$ M. PA, R, A; phosphocreatine 19, $[MgATP] = 5$, creatine kinase 1–2 mg ml⁻¹. All solutions; $[Mg^{2+}] = 1$, trimethylamino ethanesulphonic acid 100, pH 7.0, I 0.2 M, 20 °C. Scales: *a-c*, tension 100 mg, time 1 min; *d, e*, tension 84 mg, time 41 s. Tension was close to zero at the start of the records.

had been treated with cardiac and rabbit skeletal light chains produced less tension than normal both in the presence and absence of calcium. This inhibition was particularly pronounced after long incubations with light chains and no inhibition was observed when the same fibre bundle was tested before and after the addition of light chains, when full combination was achieved after incubation for 1 h.

Although it was comparatively simple to remove both scallop light chains per myosin, it proved difficult to remove exactly one light chain per myosin (by treatment with EDTA at 7 °C, which is successful with myofibrillar preparations^{2,4}). Different preparations and slightly different procedures resulted in from 0.7 to 0.9 light chains remaining. These fibre bundles did not show a complete loss of sensitivity towards calcium, that is, the tension developed in activating solution was greater than that in relaxing solution (control fibre bundles in Fig. 2*a* and Table 2). This result is difficult to interpret quantitatively because light chain

removal may not have been homogeneous throughout a bundle, so that some myosin molecules may have remained with both light chains intact. The addition of non-molluscan light chains to such fibre bundles resulted in the combination on average of more than one foreign light chain per myosin and there was a considerable variation in the amount combined (range 1.26–1.60 light chains per myosin). All the light chains tested gave a considerable restoration of sensitivity towards calcium (Fig. 2*b* and Table 2) and it is likely that the restoration was mainly due to the combination of foreign light chains to myosin molecules with a single remaining scallop light chain. The degree of restoration of sensitivity for rabbit skeletal and cardiac light chains is surprisingly high; the gel results show that a substantial number of myosin molecules must have become doubly substituted, and these molecules would have remained desensitized (Table 1). It is possible that the inhibition of tension development observed for double substitutions by these light chains reduced their contribution, resulting in an overestimate of sensitivity. Double substitution by lobster light chains had no inhibitory effect and it is noticeable that the degree of restoration of sensitivity in the single substitution experiments with lobster light chains was less than for cardiac or rabbit skeletal light chains. The full restoration of sensitivity by gizzard light chains was to be expected as these light chains confer sensitivity on their own (Table 1).

These results confirm ATPase measurements in scallop myofibrils; when both scallop light chains have been removed, sensitivity can be wholly restored by the combination of light chains from molluscan species and from vertebrate smooth muscle, but not with light chains from vertebrate skeletal, cardiac or lobster muscle³. They also confirm that sensitivity is restored in the case of a single remaining scallop light chain following the combination of light chains that do not themselves confer sensitivity towards calcium^{3,4}.

Table 1 Regulatory light chain content and calcium dependence of tension development in double-substitution experiments

Source of added regulatory light chain	No. of fibre bundles	% Sensitivity (mean $\pm$ s.d.)	Regulatory light chains per myosin (mean $\pm$ s.d.)
Control	15	0	0
Scallop striated	7	89 $\pm$ 18	1.66 $\pm$ 0.35
Spisula	2	100	1.76
Loligo syphon retractor	2	98	2.14
Mercenaria	3	95 $\pm$ 3	1.77 $\pm$ 0.10
Bovine cardiac	4	14 $\pm$ 28*	1.74 $\pm$ 0.12
Rabbit striated	9	5 $\pm$ 6	1.84 $\pm$ 0.45
Lobster tail	4	3 $\pm$ 2	1.65 $\pm$ 0.46
Chicken gizzard	6	92 $\pm$ 5	2.05 $\pm$ 0.30

Sensitivity of tension development (second column) was obtained from the ratio of the extra tension developed in activating solution (compared with the tension in relaxing solution) to the total tension developed in activating solution and expressed as a percentage. Fibre bundles were chemically skinned with 1% Brij 35 in 50 mM KCl, 5 mM EGTA, 5 mM MgCl₂, 5 mM ATP and 20 mM imidazole-HCl, pH 7.0, usually at 18–20 °C for 0.5–1 h. Light chains were removed by a 0.5–3 h incubation at 25–27 °C with 10 mM EDTA in 40 mM KCl and 20 mM imidazole-HCl, pH 7.0. Fibre bundles were combined with light chains by a 1–3 h incubation with 0.5 ml 0.1% light chain in 50 mM KCl, 5 mM MgCl₂, 5 mM EGTA and 20 mM imidazole-HCl, pH 7.0, in the presence of 1 mM freshly prepared dithiothreitol. Incubation at 4 °C gave the most reproducible results for tension measurements. Light chains were prepared as described previously⁴. Scallop, mercenaria and spisula light chain contents were determined by urea-acrylamide gel electrophoresis; lobster, loligo, rabbit skeletal, cardiac and gizzard light chain contents were obtained from 14–15% SDS-acrylamide gel electrophoresis in phosphate. Fibre bundles were rinsed with water and freeze-dried. Sample buffer (70 μ l) was added to the dried fibre bundles and incubated for 3 min in boiling water (SDS gels) or for 2 h at 37 °C (urea gels). Although the fibre bundles did not dissolve completely in the sample buffer, the light chains dissociated fully and repeated incubation with new sample buffer did not extract additional light chains. Aliquots (50 μ l) of the samples were applied on tube gels. Gels were stained with acid fast green and analysed densitometrically. Densities obtained with light chains were corrected for molecular weights and stain uptake. Regulatory light chain content per myosin was obtained from the density ratios of the regulatory and essential light chain bands. The two essential light chains of myosin are not removed by EDTA treatment², so a ratio of 1 corresponds to 2 regulatory light chains per myosin.

* Three of the fibre bundles gave low tensions.

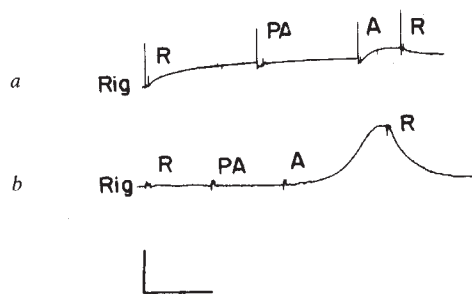


Fig. 2 Single-substitution experiments. Effect on tension development of removal of a single scallop regulatory light chain and replacement by a non-molluscan light chain. *a*, Single remaining scallop light chain (control); *b*, single substitution by cardiac light chains. Solutions as described in Fig. 1 legend. Scales: *a*, tension 50 mg, time 1 min; *b*, tension 50 mg, time 43 s.

Table 2 Regulatory light chain content and calcium dependence of tension development in single-substitution experiments

Source of added regulatory light chain	No. of fibre bundles	% Sensitivity (mean $\pm$ s.d.)	Scallop regulatory light chains per myosin (mean $\pm$ s.d.)	Foreign light chains per myosin (mean $\pm$ s.d.)
Control	7	43 $\pm$ 21	0.78 $\pm$ 0.07	
Bovine cardiac	6	96 $\pm$ 3		1.35 $\pm$ 0.08
Rabbit striated	6	88 $\pm$ 8		1.47 $\pm$ 0.11
Lobster tail	6	73 $\pm$ 17		1.33 $\pm$ 0.09
Chicken gizzard	6	98 $\pm$ 3		1.52 $\pm$ 0.12

Methods used were similar to those given in Table 1 legend except that incubation in EDTA solution was at 7 °C.

These experiments were performed at the Marine Biological Laboratory, Woods Hole and at University College London. The work was supported by PHS grant AM 15963 and a Muscular Dystrophy Association grant to A.G.S.-G. and a Programme Grant from the MRC to R.M.S. We thank the Wellcome Trust for a travel grant to R.M.S.

Received 23 November; accepted 16 June 1980.

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Formation of helical filaments by copolymerization of two types of 'straight' flagellins

Ritsu Kamiya & Sho Asakura

Institute of Molecular Biology, School of Science, Nagoya University, Nagoya 464, Japan

Shigeru Yamaguchi

Department of Biology, School of Education, Waseda University, Tokyo 160, Japan

Bacterial flagella undergo transition between several discrete types of left-handed and right-handed helical structures when exposed to acidic¹ or alkaline pH², or to mechanical force³. Calladine⁴ and ourselves⁵ have presented models to explain such polymorphism, assuming that protein subunits (flagellin) in a flagellum can be transformed into two conformations (L- and R-states) depending on the species of flagellin and on the environmental conditions. An obvious prediction from these 'two-state' models is that there should be two types of straight flagella (L- and R-types) that are made up exclusively of flagellins in either the L-state or the R-state. We have shown that straight flagella from two species of mutants, *Salmonella* SJ814 (ref. 6) and *Escherichia coli* hag 177 (ref. 7), are closely similar to the predicted R- and L-types, respectively⁵. Recently we have isolated 10 strains of straight-flagellar mutants of *Salmonella*. We show here that their flagella can also be classified into the L- and the R-types, and that copolymerization of flagellins from two heterologous types (L and R) makes discrete types of helical filaments, whereas that of homologous pairs of flagellins (L and L, or R and R) makes only straight filaments.

Ten mutant strains with a straight-flagellar phenotype (SJW 1655–SJW1664) were isolated from SJW1103, which produces normal flagella with the antigenicity of i in phase 1 and is nonflagellate in phase 2 (S.Y., in preparation). All of them are

spontaneous mutants selected on semi-solid agar plates. Flagella from these mutants were examined by electron microscopy and optical diffraction, after they had been detached from the cell body and partially purified by differential centrifugation. It was found that the diffraction pattern of the SJW1655 flagella was significantly different from those of the other nine species. Most notably, the z-coordinate of the near-equatorial layer-line (the reflection from 11 near-longitudinal rows of subunits⁶) was as large as 1/400 Å⁻¹ in SJW1655, whereas it was about threefold smaller in the rest of the mutants (Fig. 1). As the diffraction pattern of the SJW1655 flagella was closely similar to that of *Salmonella* SJ814 flagella⁶, we considered these two types of flagella to be homologous forms. The twisting of the near-longitudinal rows of subunits in the SJW1655 flagella, obtained from 34 measurements, was 7.8 $\pm$ 0.8° at the surface of the flagellum. This value was in good agreement with the twisting predicted for the R-type straight flagella⁵. On the other hand, the diffraction patterns of the other nine kinds of straight flagella were similar to that of *E. coli* hag177 flagella⁷, which have been considered to be L-type⁵, in that the spacing of the near-equatorial layer-line was about 1,500 Å. Measurements of this spacing in 31 diffraction patterns of SJW1660 flagella indicated that the near-longitudinal rows of subunits in this kind of flagella were inclined at an angle of 3.4 $\pm$ 1.0° with respect to the filament axis. Also, the other species of flagella (about 10 measurements for each) had mean values of twisting of between 2.0° and 3.4°, agreeing with the value predicted for the L-type⁵. From these results, we considered that flagella from strains SJW1656 to SJW1664 belonged to the L-type.

Flagellins from two R-type mutants (SJ814 and SJW1655) and two L-type mutants (SJW1658 and SJW1660) were mixed pairwise in all possible combinations at various protein ratios, and polymerized by the addition of fragmented flagella⁸. It was

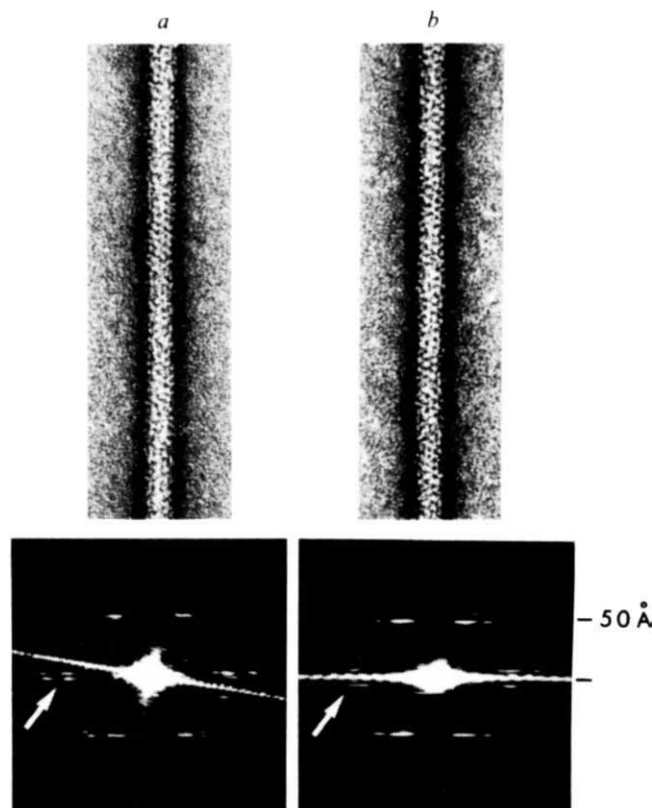


Fig. 1 Electron micrographs and optical diffraction patterns of the two types of straight flagella. *a*, The L-type from SJW1660; *b*, the R-type from SJW1655. Flagellar specimens were negatively stained with 1% uranylacetate. The diameter of the flagellum is about 200 Å. In the diffraction pattern, the near-equatorial layer-line is arrowed. Other mutants (SJW1656–SJW1659, SJW1661–SJW1664) gave diffraction patterns closely similar to that of *a*.

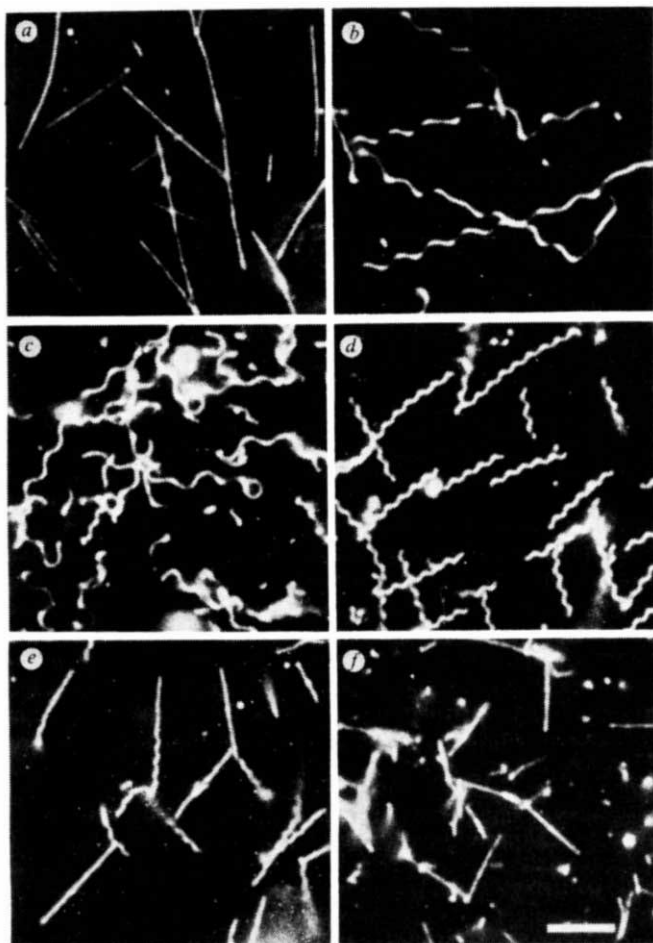


Fig. 2 The products of copolymerization of flagellins from SJW1660 (L-type) and SJW1655 (R-type) at various mixing ratios. The protein ratio of the R-type flagellin to the total monomers was: a, 0%; b, 10%; c, 25%; d, 50%; e, 90%; f, 100%. The two kinds of monomeric flagellins were prepared by the method of S.A. and Iino⁸ to give a concentration of $5.0 \text{ mg protein ml}^{-1}$ in 0.15 M NaCl . After they had been mixed at various ratios, $1/20$ to $1/8$ volume of fragmented flagella from SJW1655 (5.0 mg ml^{-1}) was added to each mixture as nuclei for polymerization. It took about 2 days for almost all the flagellin monomers to be polymerized. Essentially the same results were obtained when fragmented flagella from SJW1660 were used as the polymerization nuclei. The SJW1665 flagellin polymerized much more slowly than the SJW1660 flagellin, and for unknown reasons, it was difficult to obtain long polymers of the SJW1655 flagellin. All the photographs were taken with a dark-field microscope with an intense light source¹ after the specimens had been diluted to about 0.03 mg ml^{-1} with 10 mM sodium phosphate buffer ($\text{pH } 7.0$). Scale bar, $5 \mu\text{m}$.

found that when homologous pairs of flagellins (L and L, or R and R) were mixed, the products were always straight filaments, irrespective of the mixing ratio. However, when heterologous combinations of flagellins (L and R) were mixed, the products assumed various helical forms (Fig. 2). As in the previous experiments of copolymerization using flagellins from SJ814 and from a wild-type strain⁸, helical shapes assumed by the products were discrete. It was the frequency of appearance of a helical form that depended on the mixing ratio of the two kinds of flagellins. The frequency of a helical form depended also on the species of the two types of flagellins used. When the ratio of the R-type flagellin was less than 25% of the total monomers, in the copolymerization of the SJW1660 and the SJW1655 flagellins, the products were mostly left-handed helices, including a form resembling the wild-type flagella (normal form). As the ratio of the R-type flagellin was increased, several types of

right-handed helices appeared, including the so-called curly type. We have observed seven types of helical forms in these copolymerization experiments besides the two types of straight flagella. Morphological analyses of these helices will be reported elsewhere.

As the antigenicity of the SJ814 flagellin (1, 2) was different from that of the other kinds of flagellins used (i), we could prove that the polymers formed in the mixture of the SJ814 and one of the other species of flagellins were actually copolymers, by showing that all of them could be labelled with either anti-1, 2 or anti-i antibody (Fig. 3). The distribution of the antibodies along a filament showed a strong gradient, corresponding to the different rate of polymerization of the two kinds of flagellins. Such a gradient has frequently been observed in previous studies of copolymerization^{8,9}, and may explain the simultaneous occurrence of several types of helices in a single copolymer. We are now examining the distribution of the antibodies at a substructural level in heterologous copolymers such as shown in Fig. 3a, to see whether or not the two types of flagellin molecules are arranged with regularity. Although we have not come to a definite conclusion due to the limited resolution of electron micrographs, our impression has been that the substructural distribution is rather random. If this is found to be the case, we should assume that the flagellin molecules from either the L- or the R-type of straight flagella can be transformed into both L- and R-forms once they are incorporated into a copolymer, and that a flagellar filament has an inherent ability to organize itself into a uniform helix. At any rate, our demonstration that two types of flagellins from straight flagella copolymerize into helical

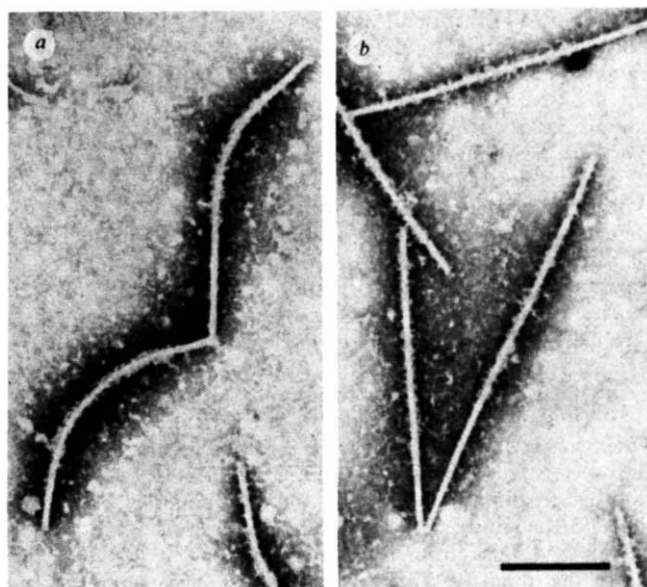


Fig. 3 Antibody staining of copolymers. Flagellins used are: a, SJ814 (R-type, antigenicity 1, 2) and SJW1660 (L-type, antigenicity i); b, SJ814 and SJW1655 (R-type, antigenicity i). Note that the filaments are curved in a, whereas they are straight in b. In each case, $1:1$ mixture of the two kinds of flagellins (5.0 mg ml^{-1}) was polymerized by the addition of $1/2$ volume of fragmented flagella from SJ814 (5.0 mg ml^{-1}). After being adsorbed onto electron microscope grids, the specimens were treated with anti-i antibody and negatively stained with 0.5% phosphotungstate ($\text{pH } 7.0$). In both of the photographs, the V-shaped portions with no antibodies attached correspond to the fragments of the SJ814 flagella used as the nuclei of polymerization. In a, the density of antibodies rapidly decreases along the length towards the tip, indicating that the SJW1660 flagellin was incorporated much faster than the SJ814 flagellin. When these specimens were reacted with anti-1, 2 antibody instead of anti-i, whole lengths of filaments were labelled, with density distributions complementary to these photographs (data not shown). Scale bar, $0.2 \mu\text{m}$.

filaments should provide strong support for the idea that flagellar polymorphism arises as the consequence of dimorphic transition of the flagellin molecules.

Received 18 April; accepted 3 June 1980.

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A conformational isomer of bovine pancreatic trypsin inhibitor protein produced by refolding

D. J. States, C. M. Dobson, M. Karplus & T. E. Creighton*

Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

* Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Refolding of the bovine pancreatic trypsin inhibitor protein (BPTI) after reduction of its three cysteine disulphide linkages can occur when the reduced protein is placed in a suitable oxidizing medium (ref. 1 and refs therein). The refolding process has been studied by trapping chemically intermediates which contain only one or two disulphide bonds (ref. 1 and refs therein). We report here that during structural studies of these intermediates by NMR, we have found that complete re-oxidation of the protein results in substantial quantities of a metastable folded species which is identical to native BPTI in its covalent bonding (including the disulphide bonds) but possesses a somewhat different conformation. The existence of such a species is supported by circular dichroism measurements on refolded BPTI². This novel form of BPTI is of considerable interest because it can be used to provide information about the folding mechanism and conformational stability of the protein.

Figure 1 shows ¹H NMR spectra of native BPTI and of BPTI which was reduced and re-oxidized fully as described in detail elsewhere³. The overall appearance of the spectrum of the re-oxidized material is similar to that of the native protein, but examination shows that two species are present. One of these has an NMR spectrum indistinguishable from that of native BPTI; the other has a slightly different spectrum and is a new species formed in the refolding process. Samples of BPTI were reduced and refolded in various conditions (using oxidized dithiothreitol rather than glutathione as the oxidizing agent and varying the pH, ionic composition and temperature of the refolding environment). In every case a mixture of two products was obtained and the novel species had the same NMR spectrum. It seems, therefore, that a conformational isomer exists which has a structure independent of the specific folding conditions, although the amount of the isomer produced does depend on them. In particular, more isomer was observed when refolding was carried out with slower rates of disulphide formation and at lower temperatures; the ratio of the isomer to native BPTI varied from 2.3 at 5 °C to 0.7 at 35 °C in the conditions described in Fig. 1 legend.

The NMR spectra of samples of the refolded material remained essentially constant when samples were left in solution for days at room temperature. Warming the solution to 37 °C for several minutes resulted in the disappearance of the resonances

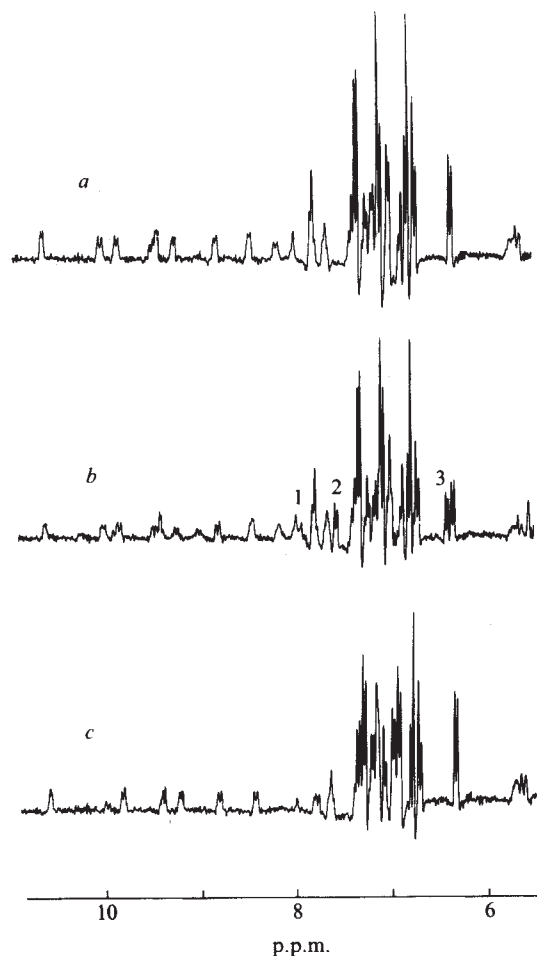


Fig. 1 Aromatic and peptide NH proton region of the 270-MHz NMR spectra of native BPTI (a), BPTI following reduction and anaerobic reoxidation with oxidized glutathione at 25 °C, pH 8.7, as described previously¹ (b), and native BPTI in which the 14–38 disulphide was reduced and the thiols blocked with iodoacetate (c). Spectra were recorded at 25 °C of 2 mM protein dissolved in D₂O at pH 6.0, and the resolution is enhanced using convolution difference¹⁹. Peaks 1 and 2 are assigned to Phe 45, and 3 to Tyr 23.

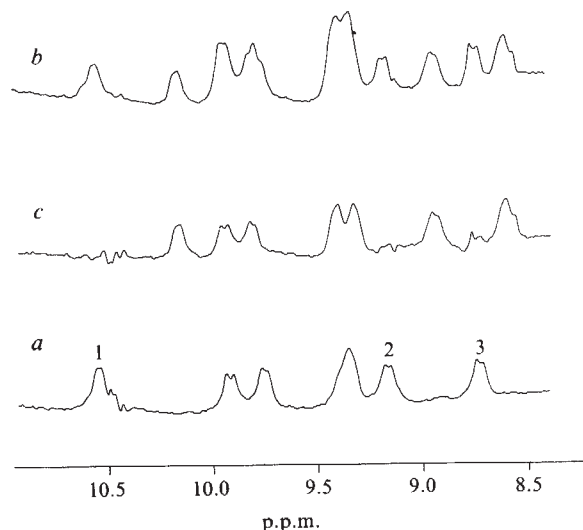
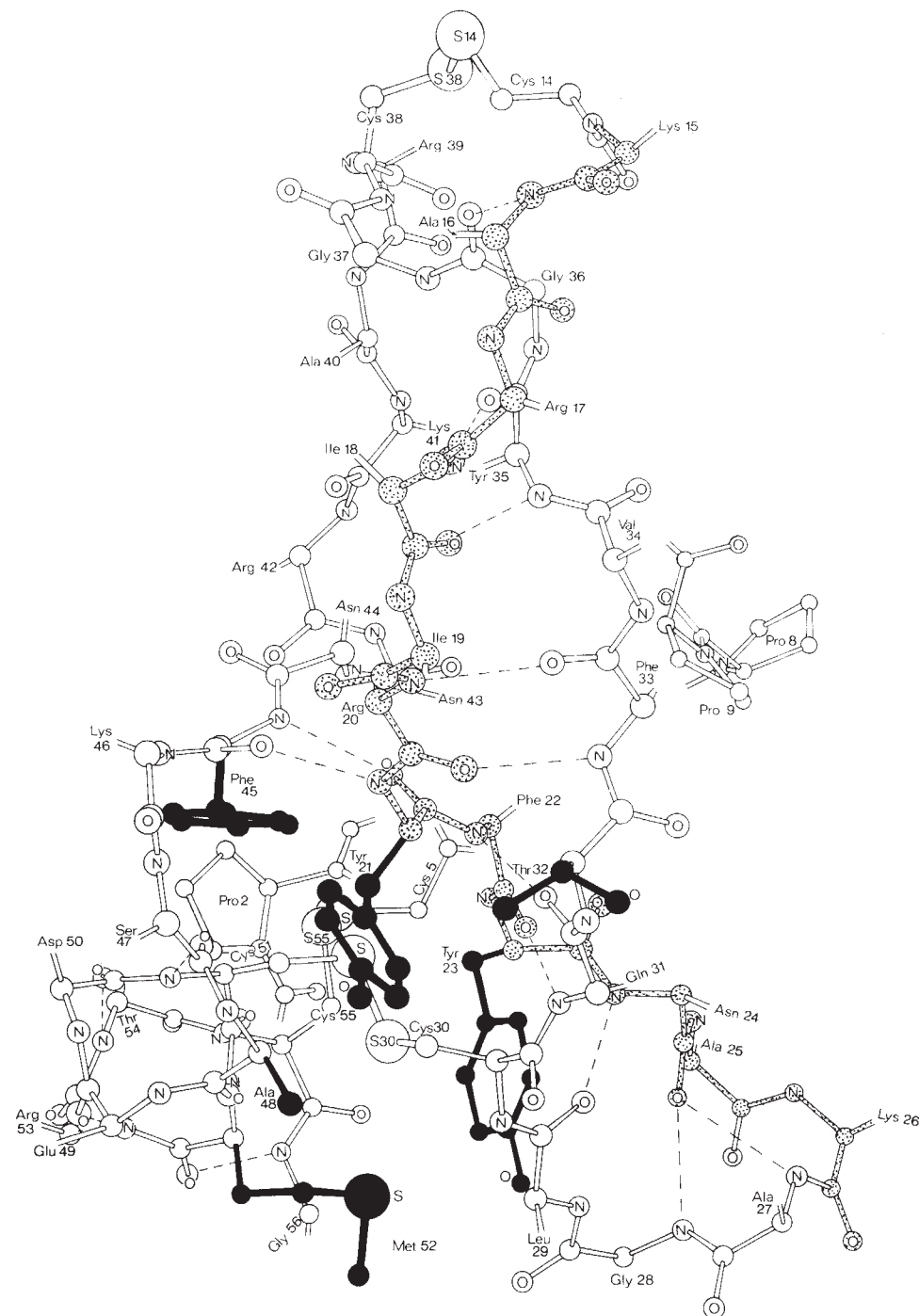


Fig. 2 Low field region of the 270-MHz NMR spectra of native BPTI (a) and BPTI refolded (b) as described in Fig. 1 legend. Spectrum c is a difference spectrum in which a fraction of a was subtracted from b to reveal the spectrum of isomeric BPTI. Peaks 1, 2 and 3 are from the peptide NH protons of Tyr 23, Tyr 21 and Gln 31, respectively⁹.

Fig. 3 Part of the crystal structure¹² of native BPTI showing the region of the protein where conformational differences exist in the metastable form. Side chains of residues whose resonances have been observed to have the largest chemical shift differences between the native and isomeric BPTI are shown in dark shading. The lightly stippled backbone is that portion which is threaded through the loop generated by the 30–51 disulphide bond.



of the isomer and an increase in the intensity of the resonances of the native protein. By following the intensities of resonances of the two species, the conversion of the isomer to the native species was found to proceed at a rate of $(3.5 \pm 0.7) \times 10^{-5} \text{ s}^{-1}$ at 37 °C and pH 6.0. Measurement of the temperature dependence of the rate gave an activation energy of $55 \pm 10 \text{ kcal mol}^{-1}$.

It is clear from these data that the refolding of BPTI results in a significant and sometimes dominant amount of a metastable species with a different NMR spectrum, and therefore a different conformation, from native BPTI. That the conversion of the metastable species to the native species takes place in conditions in which rearrangement of disulphide bonds cannot occur shows that the same disulphide bonds are present as in the native species, as had also been shown by peptide analysis⁴. Comparison of the two NMR spectra shows further that the basic structural arrangement of the native BPTI is not lost in the new species. Of particular importance is a comparison of the proton resonances of the aromatic and methyl groups, which have all been assigned in the spectrum of native BPTI^{5–7}. Most

of these resonances have the same positions (to $\pm 0.02 \text{ p.p.m.}$) in the two spectra, indicating that much of the protein conformation is essentially the same in the two species. Significant differences (shifts up to $\pm 0.1 \text{ p.p.m.}$) are found for the resonances of Tyr 21, Tyr 23 and Phe 45 in the aromatic region and of Thr 32, Ala 48 and Met 52 in the methyl region. Further information comes from the resonances of the peptide NH hydrogens which exchange slowly with solvent. In the spectrum of isomeric BPTI there are resonances within 0.05 p.p.m. of resonances in the spectrum of native BPTI for 7 out of the 10 protons whose exchange rates^{9,11} are less than 10^{-3} min^{-1} at 22 °C and pH 6.5. Three peaks, assigned to Tyr 21, Tyr 23 and Gln 31, have chemical shift values which differ in the two spectra by more than 0.1 p.p.m. (Fig. 2). The distinctive chemical shifts and long exchange rates of these protons have been attributed to their role in internal hydrogen bonding^{10,11}; most of the resonances arise from the β -sheet secondary structure, which passes through the entire molecule (see Fig. 3). The conservation in number and near conservation of chemical shifts of

these NH resonances in the two spectra indicate, therefore, that the hydrogen bonding network of native BPTI is intact in the isomeric species. In particular, it has been established¹² in native BPTI that the polypeptide chain passes through a loop produced by the existence of the 30–51 disulphide bond (Fig. 3), and the NMR data exclude the possibility that the protein chain is incorrectly 'threaded' in the isomer.

All the residues whose resonances are perturbed are rather close together, in the portion of the molecule (native structure) surrounding the 30–51 and 5–55 disulphide bonds, which are near to each other (Fig. 3). The major conformational differences between native and metastable BPTI therefore lie in this part of the structure.

It is useful to compare this conclusion with the results⁵ from studies of a derivative of BPTI with one of the disulphide bonds, 14–38, reduced. The spectrum of this species is shown in Fig. 1c. There are no large shifts in any of the resonances and those with small shifts arise from residues in the part of the molecule near to the broken 14–38 bond, notably Thr 11 and Tyr 35. There are negligible shifts in the resonances perturbed in the isomeric BPTI, indicating that the conformations of the isomeric BPTI and the 14–38 reduced species are altered in different regions of the structure.

The simplest explanation for the existence of the isomer would be that it is a normal intermediate in the final step of the refolding process; that is, when the last disulphide bond (14–38) is formed, the isomeric conformation is produced which then goes on to generate slowly the native conformation. This possibility is unlikely because a significant proportion of BPTI in the native conformation is formed when refolding (which takes only minutes) is carried out at temperatures so low (<25 °C) that the metastable isomeric species converts to the native species only over a period of days. Thus, it is more likely that the two forms of folded BPTI arise from alternative parallel pathways. Further, it has been found that re-oxidation of the 14–38 reduced species results in BPTI with a spectrum identical to that of the native species, so that it is unlikely that the conformational differences between the isomers arise in the formation of the 14–38 bond.

To investigate possible origins of the alternative pathway in earlier parts of the refolding reaction, the one- and two-disulphide bond intermediates have been examined. The conformations of these, other than the one with only the 14–38 bond in the reduced state, are significantly different from that of native BPTI, with a much less well defined globular structure, particularly in the one-disulphide species. This is in essential agreement with conclusions from immunochemical analysis of these intermediates¹³. It seems possible, therefore, that the conformational isomer arises from the same disulphide bond formation sequence as native BPTI but has a difference in structure which arises because of the stability of this difference in an early intermediate. This could be due to the existence of a *cis* rather than the *trans* conformer found for all four proline residues in native BPTI (Pro 2 is close to the region of the observed structural changes)^{14,15}, a different conformation around the 30–51 or 5–55 disulphide bond, or some other difference in a main-chain or side-chain dihedral angle. The formation of the 14–38 bond, or some earlier step, could then 'lock in' the conformational difference. Alternatively, the two different folded species could result from pathways in which the sequences of disulphide bond formation are different. For example, in the initial stages of folding several pathways are known to exist and to result in the formation of different two-disulphide species, which then rearrange to the 30–51, 5–55 species before complete refolding. It is interesting in view of the results reported here that the relative stabilities of different two-disulphide species have been found to be temperature dependent (T.E.C., unpublished results).

The present results are of significance for an understanding of the mechanism of protein folding and for the analysis of the determinants of protein conformation. Of primary interest is the demonstration that two similar globular structures can exist; even in a protein as small as BPTI a specific structure can be

formed during folding that is not at the free energy minimum^{16,17}. Further, the activation energy for the transition from the metastable to the native species is high; it is of the order of the highest activation energies measured for exchange of the backbone amide hydrogens of native BPTI¹⁰. This suggests that, although the conformational difference between the two species is localized, the transition involves a significant disruption of the structure. If the specific element that distinguishes the two structures is a dihedral angle rotation, conformational constraints would have to be the source of the high activation energy. This is analogous to the tyrosine and phenylalanine ring 'flips' for which the observed activation energies of up to 25 kcal mol⁻¹ are much larger than the internal rotation barrier^{7,18}.

It will be of considerable interest to use the existence of this conformational isomer to elucidate the structural and dynamic aspects of folding transitions in proteins.

We thank Bayer AG for a gift of BPTI (Trasyolol). The NMR experiments were carried out at the NMR Facility for Biomolecular Research, Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology. The NMR Facility is supported by grant RR0995 from the Division of Research Resources of the NIH, and by the NSF under contract C-670. This work is also supported in part by grants GM26272 and EY00062 from the NIH.

Received 18 February; accepted 5 June 1980.

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A diffusion–collision–adhesion model for the kinetics of myoglobin refolding

F. E. Cohen*, M. J. E. Sternberg & D. C. Phillips

Laboratory of Molecular Biophysics, Department of Zoology, South Parks Road, Oxford OX1 3PS, UK

I. D. Kuntz & P. A. Kollman

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143

There are two distinct experimental and theoretical problems of protein folding: the thermodynamic issue of characterizing the folded state, and the kinetic question of the path between the unfolded and native states¹. Here we consider the second question and present a diffusion–collision–adhesion model for the folding of the α -helical protein myoglobin. In particular, we consider the fast refolding species of the unfolded state and ignore the slow transition between unfolded states that has been attributed to proline isomerization².

* Present address: Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California.

Baldwin³ and Karplus and Weaver^{4,5} have considered the experimental and theoretical evidence as to the probable rate-limiting step during (fast) folding. They agree that the formation of a local structure by a random search is unlikely to act as a nucleus for subsequent rapid folding. Instead, they opt for intermediate control in which the relative stabilities of the intermediates determine the pathway(s) and the rate of folding. Local structures (for example, α -helices) of transient stability diffuse together, collide and then adhere (pair) together. There is no requirement for an obligatory intermediate and folding may proceed along several paths. The intermediates for myoglobin are assemblies of α -helices as suggested by Ptitsyn and Rashin⁶.

Rate constants for the steps are derived from the model of Karplus and Weaver^{4,5}: collision, not adhesion, is the rate-limiting step. Detailed stereochemical information about helix packing in myoglobin is taken from the work of Richards and co-workers^{7,8}. The folding follows intermediate control, shows the cooperative behaviour that has been observed experimentally^{9,10}, and we find several paths linking the unfolded and native states. A second calculation considers N-terminal folding that may occur during biosynthesis^{11,12}. In our model, apomyoglobin is considered as six fluctuating α -helices (ABEFGH). Initially, pairs of α -helices collide to form a cluster. Each cluster can grow by docking either with one other α -helix or with another cluster that contains two or more α -helices. We allow only the five strongest helix/helix interactions (AH, BE, BG, GH, FH)⁷ that are observed in the folded myoglobin. This implies that the interactions that are dominant in the final structure are important in the folding pathway and that incorrect helix pairings do not significantly interfere with the rate of formation of native intermediates.

In the model of Karplus and Weaver^{4,5}, the time required for the interaction of two helices is

$$\tau = \frac{1}{\beta} \left(\frac{l \Delta V}{DA} \right) \quad (1)$$

where l is the characteristic length, ΔV is the volume of finite diffusion space, D is a diffusion coefficient ($\sim 1 \times 10^{10} \text{ Å}^2 \text{ s}^{-1}$), A is the area of the target surface and β is the fraction of time that both segments are helical when they collide.

To calculate rate constants for the condensation of two helices, we assume that ΔV is the space between two concentric spheres. The outer radius is determined by

$$r_{ij}^{\text{ext}} = 1.5H + 3.3C \quad (2)$$

where i and j are the residues central to the helix-helix interaction⁷, and H and C are the number of residues between i and j in helical and coiled conformations, respectively. The inner radius, r_{ij}^{pack} , is taken as the length of the segment which is mutually perpendicular to the axes of the interacting helices^{7,8}.

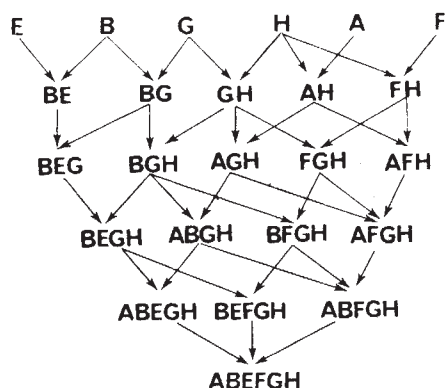


Fig. 1 Possible folding paths for myoglobin. Only the addition of single helices to preformed clusters is depicted. Condensations between preformed clusters, for example, $BE + GH \rightarrow BEGH$, are omitted for the sake of clarity.

Table 1 Parameters for the diffusion-collision equation

Interaction	Average potential contact area loss on association* $A = (A_i + A_j)/2 (\text{Å}^2)$	$r_{ij}^{\text{pack}} (\text{Å})$	$r_{ij}^{\text{ext}} (\text{Å})$
G111-H135	185	10.5	25.05 (17.55 $\pm$, 19.05 $\pm$, 17.55 $\pm$)
A10-H134	170	8.5	189.3 (95.3 $\pm$, 26.0 $\pm$, 118.2 $\pm$, 142.05 $\pm$, 15.2 $\pm$)
B25-E65	145	7.5	50.70 (43.20 $\pm$, 24.00 $\pm$)
F90-H142	125	8.5	66.90 (54.15 $\pm$, 12.90 $\pm$)
B28-G110	155	8.5	120.00 (45.45 $\pm$, 112.50 $\pm$, 12.9 $\pm$, 89.40 $\pm$)

* From Richmond and Richards⁷.

$\pm$ AH formed; $\pm$ BE formed; $\pm$ BG formed; $\pm$ FH formed; GH formed.

If the target area for the interaction is the average of the potential accessible areas lost when the two helices pack (A_i and A_j)⁷, then equation (1) becomes

$$\tau = \frac{\frac{1}{2}(r_{ij}^{\text{ext}} + r_{ij}^{\text{pack}}) \times \frac{4}{3}\pi((r_{ij}^{\text{ext}})^3 - (r_{ij}^{\text{pack}})^3)}{\beta 10^{10} \frac{1}{2}(A_i + A_j)} \quad (3)$$

Clearly, r_{ij}^{ext} is a function of the degree and nature of the condensation of the molecule. For example, the volume of diffusion space available for the FH interaction is decreased if GH has been formed (see Table 1).

In our model, α -helices that have correctly paired do not dissociate. Clearly, proteins denature and so a helix-helix pair can dissociate. However, in conditions where the folded structure is stable, dissociation will be slower than association. If the stability of the native structure is indicative of the stability of various intermediates, dissociation is probably negligible. Moreover, the rate of dissociation is a complex function of the solvent and temperature that remains undefined.

The only adjustable constant in this analysis is ρ , the probability that a potential helical segment will be helical when it interacts. For a pair of fluctuating helices, $\beta = \rho^2$; for one fluctuating helix packing against a solid cluster, $\beta = \rho$; and for two solid clusters packing, $\beta = 1$. In the two simulations that follow, $\rho = 0.002$. Smaller values of ρ decrease the cooperativity of the folding transition and larger values decrease the lag time for renaturation. Changes in ρ had little effect on which intermediates were observed, although the half lives of these species were affected.

In our model of myoglobin, there are 24 states (see Fig. 1). There exists a matrix of 24×24 second order rate constants which define a system of simultaneous second order differential equations. As a closed form solution to this problem does not exist in general, the rate equations are solved iteratively with time steps of 25 μ s. Figure 2a shows the exponential behaviour of the concentration of the final state. The concentration begins to level off after 2 ms and slowly goes to completion. Two intermediate states are significantly ($>5\%$) populated: FGH and ABFGH. The lag phase, although noticeable, is small. This is undoubtedly because the initial steps in this simulation overwhelmingly favour products. In the refolding of ribonuclease A in 50% methanol at temperatures less than -20°C , a lag in the absorbance change due to the burial of tyrosine residues is observed. The lag lasts approximately 100 s at pH 6.0, -25°C (A. L. Fink and B. Lustig, personal communication).

In an attempt to simulate biosynthesis, the initial concentrations of the isolated helices were repeated. Thus, the concentration of H was 0.0 until the concentration of G reached 1.0. The rate of synthesis was such that a myoglobin chain was made in 1.2 s. BE is the first intermediate to appear, followed by the production of BEG, BEGH and then the native state (see Fig. 2b).

Rescue of a splicing defective mutant by insertion of an heterologous intron

Peter Gruss & George Khoury

Laboratory of Molecular Virology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

It is now widely accepted that the primary transcripts of many eukaryotic genes contain intervening sequences (introns)¹⁻³. These introns, which vary considerably in length, have been found in both coding and noncoding regions⁴⁻¹⁵. They are removed from the primary transcript in one or more steps by a process called RNA splicing¹⁶. The role of RNA splicing, the size and position of introns and the signals which modulate the splicing process are under intense investigation. These studies have been greatly facilitated by the use of viral mutants or viral-eukaryotic recombinant molecules. The SV40 system has been of particular value in elucidating certain genetic elements which are critically involved in the splicing process¹⁷⁻²¹. Further insight into the biological significance of the splicing process has come from a mutant from which precisely one intron has been removed (intron-minus mutant, d1-2350). This deletion mutant has the potential to circumvent the need for splicing. However, the inability of d1-2350 to accumulate late viral transcripts indicates that splicing is a requirement for the biogenesis of stable mRNA²². This finding is supported by studies using the mouse β^{maj} globin gene inserted into SV40. The inserted portions contained either the complete globin gene²³ or a segment encompassing the intron plus the entire 3' end of the gene²⁴. As polyadenylation is known to precede splicing^{2,3}, the possibility exists that every transcript possesses sequences near the 3' end that are directly involved in the splicing process. We report here that, to test this possibility as well as to pursue the question of whether the splicing event and/or defined sequences in the intron are required for the biogenesis of mRNAs, we inserted an isolated intron lacking its own genomic 5' and 3' ends into the intron-minus mutant previously described. The experimental protocol provided for obtaining both possible orientations of the insert relative to the late genomic region. Investigation of the transcriptional products indicated that stable mRNA was produced only by the mutant containing the intron in the sense orientation.

There are, however, a few reported examples of abundant unspliced cytoplasmic mRNA. These include the histone mRNAs³⁸ (which also differ from most other eukaryotic transcripts in having an abbreviated or absent 3'-polyadenylation signal) and the messenger for adenovirus polypeptide IX³⁹. Whether these examples are fundamentally different from the majority of eukaryotic genes studied thus far, or if there is a special category of unspliced stable transcripts remains to be determined.

The *in vitro* construction of the recombinants is outlined in Fig. 1. An *Eco*RI fragment containing the entire chromosomal portion of the mouse β^{maj} globin gene^{15,25} was cloned at the *Eco*RI site in pBR322. This DNA was cleaved with *Hind*II and a fragment 573 nucleotides long was isolated by gel electrophoresis. This fragment represents the small intron (intron 1) of mouse β^{maj} globin chromosomal DNA plus some flanking sequences, but lacks the 5'- and 3'-terminal sequences of the β^{maj} globin gene. An enzyme which conveniently cuts at a single site present in the late region of the intron-minus mutant (d1-2350) is *Hpa*II. This recipient site is located in the leader region of d1-2350, 221 nucleotides upstream from the donor splice junction^{22,26}, for the SV40 16S mRNA. The cleavage of d1-2350 DNA with *Hpa*II thus results in a linearized DNA containing 'sticky' *Hpa*II ends. To obtain efficient ligation of the globin intron fragment harbouring 'blunt' *Hind*II termini to the linearized DNA, the *Hind*II ends were converted to *Hpa*II ends by the addition of synthetic oligonucleotides (linkers)²⁷. Liga-

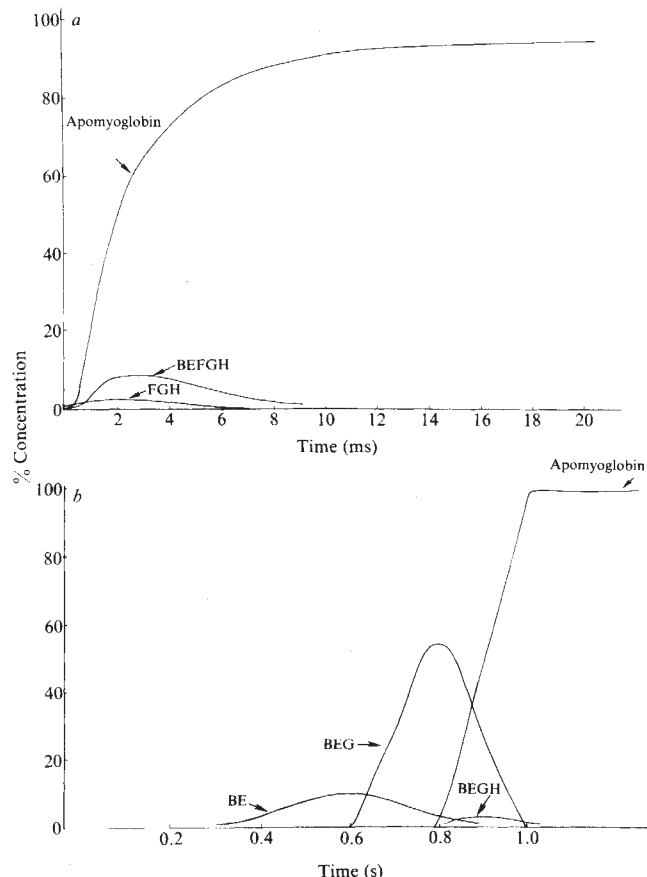


Fig. 2 *a*, Simulated renaturation kinetics of apomyoglobin as a function of time. The concentrations of intermediates which are significant are also plotted. *b*, Simulated folding kinetics of apomyoglobin during biosynthesis. The time taken to synthesize a helical segment is 0.2 s.

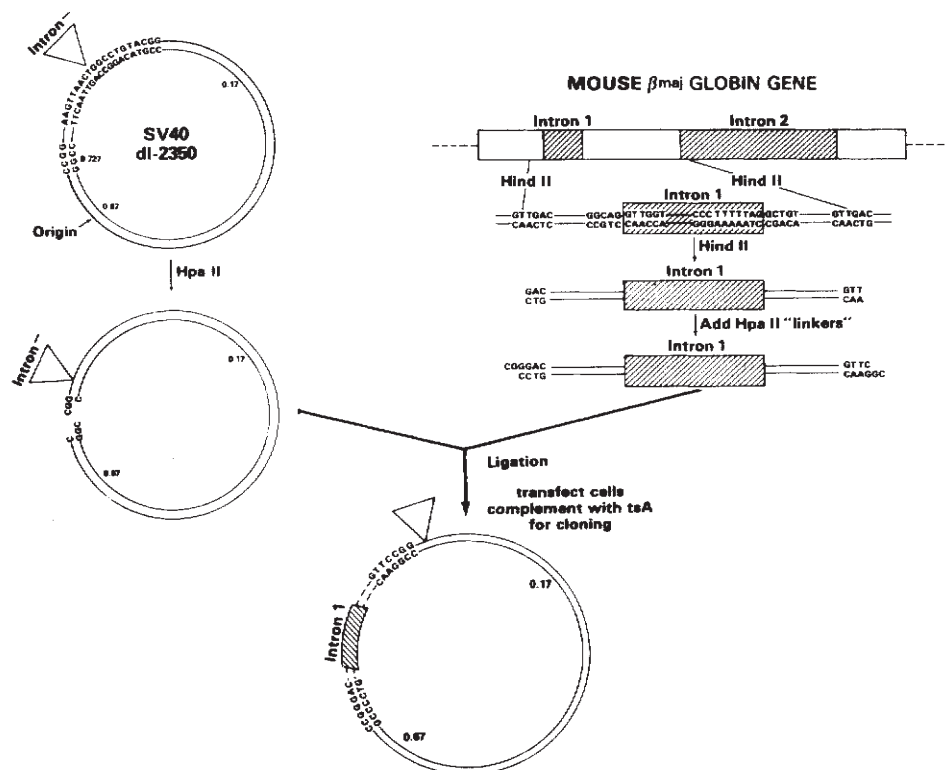
In these two simulations, two distinct sets of intermediates were significantly populated. Thus, we suggest that folding can occur through many alternative pathways which ultimately converge to the native structure. Cooperativity results from the decrease in the volume of diffusion space as a function of molecular organization. In principle, the intermediates of renaturation could be trapped and identified in a stop-flow experiment. Alternatively, the sites of helix-helix interaction could be chemically blocked to inhibit the completion of the folding and the intermediates created could then be characterized. Studies by Zabin and co-workers suggest that intermediates in biosynthesis could be characterized by immunological probes¹².

This model assumes that no mismatched pairs of hydrophobic patches on the surface of the α -helices are stabilized. A more realistic assumption would be that mismatched helix-helix pairs could tautomerize to a correct pairing. These tautomerizations would have low activation barriers, as the individual helical conformations have been stabilized and the volume of diffusion space is reduced. This tautomerization mechanism might explain Creighton's finding¹ that there is an obligatory mismatched disulphide bridge on the pathway joining the unfolded and native states of pancreatic trypsin inhibitor.

Received 14 March; accepted 9 June 1980.

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Fig. 1 Insertion of mouse β^{maj} globin intron 1 into SV40 d1-2350. Plasmid DNA (pBR322) containing the active mouse β^{maj} globin gene^{15,25} insert was cleaved with *Hind*II and a fragment of 573 nucleotides representing the entire intron 1 plus some flanking sequences was purified. This DNA (10 μ g) was blunt-end ligated to 2.5 μ g of *Hpa*II decamer linkers (CCGGATCCGG from Collaborative Research) in conditions exactly as described by Maniatis *et al.*²⁷. The DNA containing *Hpa*II linkers was purified away from unligated linkers by gel electrophoresis and subsequently cleaved with *Hpa*II. A second electrophoresis step was used to purify the DNA containing sticky *Hpa*II ends. In parallel, SV40 d1-2350 DNA (4.5 μ g) was reacted with *Hpa*II, extracted with phenol and dialysed. The globin fragment containing *Hpa*II ends (1.2 μ g) was ligated to d1-2350 DNA (4.5 μ g) in a total volume of 100 μ l in a buffer (50 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 20 mM dithiothreitol, 60 μ M ATP) using 2 units T4 DNA ligase (Biolabs). The reaction mix was incubated for 1 h at 15 °C, then further reacted for 16 h at 4 °C. The ligated recombinants were separated from unligated material by agarose gel electrophoresis. Purified recombinant DNA was used in an infectious centre assay⁴⁰ and complemented with SV40 tsA. Many plaques were picked and used to grow viral stocks in secondary African green monkey kidney cells⁴⁰.



tion was subsequently carried out using a twofold molar excess of globin DNA over the d1-2350 recipient DNA. As *Hpa*II termini were present on every end, the insertion of the globin intron can occur in both possible orientations relative to the late transcription unit. The ligated DNA products were transfected into secondary AGMK cells using the facilitating agent DEAE-dextran^{28,29}. We used tsA, a helper virus defective in early gene function at non-permissive temperature (40 °C)³⁰, to complement the ligated DNA product by providing the missing gene functions for the late viral structural proteins VP2, VP3 and VP1. The decision to use tsA rather than tsB (which is temperature sensitive for VP1) was based on three critical points: (1) As tsA provides an intact late region, it non-selectively complements all mutants defective in late function(s). The tsA mutant should be capable of complementing our ligated recombinant molecules, even if no stable mRNA or protein is produced after insertion of an intact intron. (2) Regardless of the orientation of the inserted globin intron relative to the late transcription unit, tsA should complement the recombinant molecule. (3) The chromosomal β^{maj} globin DNA fragment providing the intron also contains some flanking sequences from the globin gene. Close inspection of these sequences²⁴ reveals the presence of six ATG triplets. Should these triplets serve at the RNA level as translation initiation codons, they would produce proteins which differ from VP1, and would not complement tsB.

With the help of tsA, many clones were obtained and screened for the presence of globin DNA sequences (see Fig. 2). A plasmid carrying the entire mouse β^{maj} globin gene was cleaved with *Sac*I and the two resulting fragments were separated on an agarose gel and subsequently transferred to nitrocellulose filter paper³¹. The filter was cut into small (3 mm) strips and hybridized to ³²P-labelled DNA from cloned recombinants. As an example (Fig. 2a, c, d), three mutant clones hybridize exclusively to the small (1.15 kilobase) fragment representing intron 1 and the 5' region of the β^{maj} globin gene. One cloned mutant (Fig. 2b) seems not to contain a globin-specific insert.

The entire DNA sequence of SV40^{32,33} and mouse β^{maj} globin is known. Thus, our physical characterization of cloned recombinant DNA could be limited to a few restriction endonucleases so as to determine the orientation of the insert. A combination of cleavage by *endo R* *Ava*II and *endo R* *Bam*HI, and subsequent size determinations of the fragments by poly-

acrylamide gel electrophoresis, allows an identification of the orientation of the inserted globin intron relative to the late transcription unit (data not shown). Two classes of recombinants were obtained and are schematically outlined in Fig. 3. In the case of the recombinant d1-2350-I, the globin intron is inserted in the sense direction, whereas in d1-2350-II the intron is orientated in the antisense direction.

The structure of the recombinant molecules suggests that even after the removal of the intron by a potential splicing reaction, a stable transcript should retain a total of 456 nucleotides (intron flanking sequences) derived from the mouse β^{maj} globin gene. Thus, an mRNA from the recombinant template is expected to increase in size by this amount relative to 16S mRNA transcribed from the tsA helper template. Should unspliced recombinant mRNA be present, the size increase would be 456 + 116 = 572 nucleotides. To identify such a hybrid mRNA and to distinguish between spliced and unspliced transcripts, cytoplasmic polyadenylated RNA was prepared from AGMK cells infected with the recombinants containing the

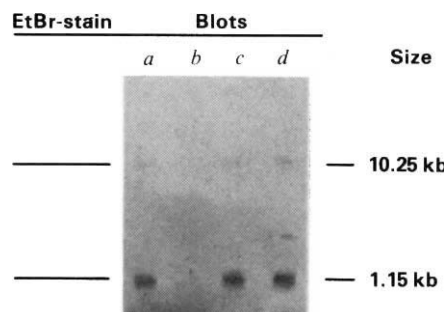


Fig. 2 Screen for recombinants containing the globin insert. DNA was prepared from a plasmid containing the entire mouse β^{maj} globin gene. This DNA was cleaved with *Sac*I, an enzyme generating two fragments. The smaller fragment (1.15 kilobases (kb)) represents the 5'-end portion (including intron 1) of the gene, the larger fragment (10.25 kilobases) contains the sequences of the 3' end. This cleaved DNA (20 μ g) was electrophoresed into a 1.4% agarose gel (in 0.04 M Tris pH 7.8, 0.005 M sodium acetate, 0.001 M EDTA) and subsequently blotted on to a nitrocellulose filter paper³¹. The filter paper was cut into strips 3 mm wide. Each strip was hybridized to a ³²P-labelled DNA preparation obtained from cells infected with the different mutant stocks³⁵. Mutants a, c and d contain the globin-specific insert, whereas mutant b does not have any detectable globin-specific sequences. EtBr, Ethidium bromide.

intron in either the sense or the antisense direction. Aliquots of both RNA preparations were electrophoresed in duplicate through an agarose gel containing methyl mercury hydroxide and subsequently transferred to diazotized benzyloxymethyl (DBM) paper³⁴. One track of each preparation was hybridized to ³²P-labelled SV40 DNA; the second track was hybridized to ³²P-labelled mouse β^{maj} globin DNA. Figure 4 presents the results. In the right-hand panel of the gel, RNA from the two recombinants (d1-2350-I and d1-2350-II) was hybridized to ³²P-labelled SV40 DNA. In both tracks two bands appear in similar positions. These positions are identical to those for the 16S and 19S mRNAs of the SV40 control (not shown). Thus, these classes apparently represent RNA derived from the tsA helper genome. In the track containing RNA from the recombinant which has the globin intron in the sense direction, an additional band (2,050 nucleotides) appears. This class of mRNA is 450 nucleotides larger than 16S mRNA. In addition to SV40 homologous sequences, this class of RNA alone also has sequences which are homologous to mouse β^{maj} globin DNA (Fig. 4, left panel). These observations indicate that the insertion of an intron in the sense direction is sufficient for activation of a late spliced SV40-containing mRNA. It seems that mouse β^{maj} globin intron 1 and its flanking nucleotides are functional as a splice signal in heterologous genes even in the absence of its original 5'- and 3'-genomic end.

Split genes, containing introns which are not represented in their terminally processed RNA, are unique to eukaryotes. The precise removal of the intron takes place at the RNA level¹⁻³. It has been suggested that the splicing mechanism is required not only for the removal of the intron but also for stabilization of the RNA^{17,18,22,23}. In the present study we have examined directly the role of an isolated globin gene intron and its flanking nucleotides inserted into a previously constructed SV40 mutant lacking precisely the intervening sequence of 16S mRNA (intron-minus mutant d1-2350)²². A principal experimental difference between this study and previous reports²³ is the rescue of stable SV40 RNA from the splicing defective mutant d1-2350 by insertion of an intron derived from a heterologous gene. As an intron source we selected a restriction enzyme fragment from the mouse β^{maj} globin gene^{15,25}, representing the entire intron 1 plus some flanking sequences. However, the 5'- and 3'-terminal sequences of the globin gene are not retained. Construction of the mutant involves insertion of globin gene intron 1 into the late region (0.72 map unit) of d1-2350. The site of insertion is 221 nucleotides upstream from the original splice junction for 16S mRNA. Thus, the location of the inserted intron differs from the original position of the intron for 16S RNA. Although the experimental protocol for the insertion

allowed ligation in both possible orientations relative to the late transcription unit, only the presence of an intron in the sense direction rescued stable mRNA. This confirms the earlier observation by Hamer and Leder that the polarity of a genomic fragment consisting of the 3' end and large β^{maj} globin intron, relative to the SV40 transcription unit, was critical to the splicing of the hybrid mRNAs²³. A correctly orientated intron can be removed in a splicing process, as indicated by the size of the resultant chimaeric RNA. It should be emphasized, however, that some mouse flanking sequences for globin intron 1 are preserved in these recombinant molecules and may be required for efficient splicing. Recent studies have suggested that small nuclear RNA (snRNA) molecules may serve as a 'guide' or strut in aligning pre-mRNA sites for splicing^{35,36}.

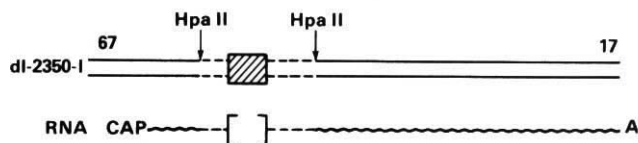
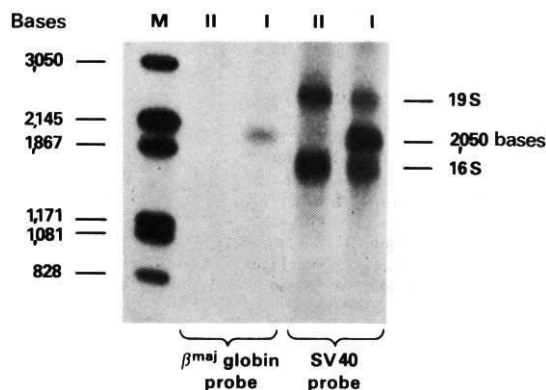


Fig. 4 RNA analysis. Cytoplasmic RNA from the recombinants was prepared 48 h after infection of secondary AGMK cells with 10 plaque-forming units per cell. Cells were treated with 0.5% NP40, 0.14 M NaCl, 0.01 M Tris pH 7.5 and 0.0015 M MgCl₂ (ref. 41). Poly(A)-containing RNA was selected by chromatography on an oligo(dT)-cellulose column. Aliquots of the RNA preparations from d1-2350-I (sense insert) and d1-2350-II (antisense insert) were electrophoresed in a 1.2% agarose gel containing methyl mercury hydroxide. Electrophoresis and subsequent transfer of the RNA to DBM paper were as described by Alwine *et al.*³⁴. The resulting imprints were preincubated and hybridized with 200,000 c.p.m. of either a ³²P-labelled SV40 DNA or a ³²P-labelled plasmid DNA containing the entire mouse β^{maj} globin gene. Both probes were made by nick translation and had a specific activity of 10⁸ c.p.m. per μ g DNA. As a size marker, ³²P-labelled restriction enzyme fragments of the indicated sizes were electrophoresed in a parallel track. The bottom portion of the figure outlines an interpretation of the data (the transcript deriving from the recombinant d1-2350-I, sense insert).

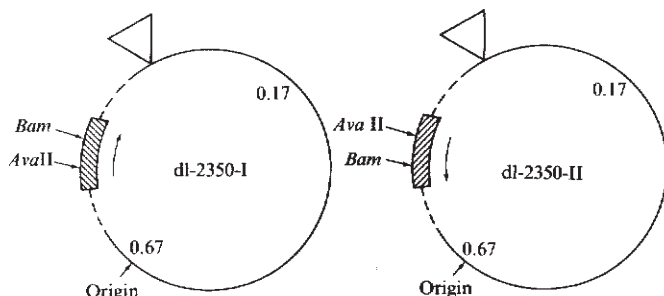


Fig. 3 Physical map of the recombinant DNA. For the establishment of a physical map, we concentrated on a determination of the orientation of the insert relative to the late transcription unit. A combination of *Ava*II and *Bam*HI endonucleases enabled us to determine the orientation by sizing the fragments. Both possible classes of recombinant give rise to six specific fragments, using these particular enzymes. Four out of those six fragments were expected to be identical (1,580, 1,006, 519 and 185 base pairs). The size of the remaining two fragments in the case of d1-2350-I (sense direction) was 867 and 710 base pairs, whereas the insert in the antisense direction (d1-2350-II) yielded two fragments of 987 and 590 base pairs. The analysis was carried out using 4% polyacrylamide gels (acrylamide = bis, 20:1) for electrophoresis in a 0.04 M Tris pH 7.8, 0.02 M sodium acetate, 0.001 M EDTA buffer.

The globin sequences flanking the inserted intron contain six ATG triplets, which are potential candidates for translational initiation in each reading frame. All precede the actual AUG triplet for initiation of VP1 translation. Attempts to clone these recombinant molecules using tsB in parallel experiments did not result in any plaques containing the recombinants. This observation confirms the data of others³⁷ suggesting that the first available AUG is in most cases preferentially used as the initiation signal for translation. Therefore, VP1 cannot be produced in amounts sufficient to complement SV40-tsB.

The flanking sequences which are preserved do not contain either the 5' or 3' end of the globin gene. Thus, it seems unlikely that either end specific to a particular mRNA is directly involved in splicing. Furthermore, the lack of obvious homology between SV40 and globin 5' or 3' ends suggests that no similar sequence is likely to be provided by SV40. The comparison of SV40 late introns with globin intron 1 did not show extensive homology, with the exception of consensus sequences at the splice junctions. Nevertheless, the insertion of the globin intron resulted in the reconstitution of stable RNA. This experimental result

provides strong support for the idea that the splicing event is coupled to the stability of the RNA. Accordingly, defined intron sequences seem less likely to be involved in a positive transcriptional regulation event. Thus, while the polarity of the intron is critical to its function, the species of origin and the relative position in the mRNA are not. These observations suggest that introns represent functional elements in the generation of certain stable mRNAs.

We thank P. Leder for providing the cloned mouse β^{maj} globin gene, M. König and J. Duvall for technical assistance, and C.-J. Lai, J. Alwine, R. Dhar, S. Segal and B. Dunn for helpful discussions. P. G. is a recipient of a grant of Deutsche Forschungsgemeinschaft.

Received 3 April; accepted 16 May 1980.

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being between the B-form (low salt) and the Z-form (high salt)¹. We now analyse the possibility that such transitions could occur in susceptible sequences in physiological conditions as a consequence of the torsional stresses imposed by superhelicity. As *in vivo* DNA commonly occurs in a negatively supercoiled, hence underwound, state, these transitions could serve important biological functions. Both thermodynamic and statistical mechanical theories of stress-induced two-state transitions have been developed previously^{5,6}. Here we apply these theories to transitions between the B-form and the Z-form in regions of appropriate base sequence. Assuming that the unstressed duplex is entirely B-form, we show that when the molecule is constrained to be underwound, susceptible regions may transform to the Z-form, thereby absorbing most of the torsional deformation. Interestingly, this transition is relatively independent of temperature.

Consider a linear segment containing N base pairs, of which N_Z sequential pairs may undergo the B–Z transition. Let this segment be twisted by q turns away from its unstressed (hence totally B-form) shape. This torsional deformation will be partitioned between local transitions to the Z-form and smooth twisting in both the B- and Z-form regions so as to minimize the total conformational free energy involved. Denote the number of base pairs per helix repeat in the unstressed B-form by $A_B = 10.4$ and in the Z-form by $A_Z = -12$ (the negative sign deriving from the presumed opposite handedness of the two forms). Let τ_B (and τ_Z) denote the twist rate at which the B-form (and Z-form) regions are deformed away from their unstressed shape. If n bases occur in the Z-form, then

$$\tau_B = 2\pi[q + (n/A) - (n\tau_Z/2\pi)]/(N - n) \quad (1)$$

where $1/A = 1/A_B - 1/A_Z$. The conformational free energy associated to this torsionally deformed structure is given by

$$F(n, \tau_Z) = a + bn + \frac{nC_Z\tau_Z^2}{2} + \frac{C_B}{2(N - n)} \left[2\pi \left(q + \frac{n}{A} \right) - n\tau_Z \right]^2 \quad (2)$$

Here, C_Z and C_B are the torsional stiffnesses of the Z- and the B-form structures, b is the free energy required to change one base pair from B to Z and a is the extra free energy needed to initiate the transition in a region. Although C_Z is not known, it is reasonable to assume that $C_Z \approx C_B$. Stable conformations occur at those values of n and τ_Z which minimize the free energy of equation (2)⁵. It follows that the segment must be underwound

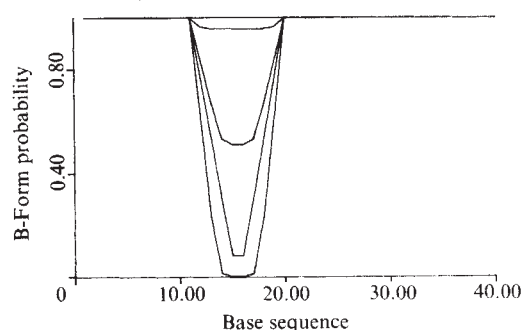


Fig. 1 The probability of being in the B-form is computed for a sequence of $N = 80$ base pairs, of which $N_Z = 8$ consecutive pairs are susceptible to transition to the Z-form. For clarity, the transition profiles of a sequence of 40 base pairs containing the susceptible region are plotted at various values of the torsional deformation q . First, when $q = 0$ all base pairs are in the B-form with probability 1. The other four curves are for $q = -0.38m$, $m = 1, 4$. As the amount of negative torsional deformation increases, so does the probability of susceptible pairs transforming to Z-form. The parameters used in this calculation are $T = 298$ K, $\sigma = 1.36 \times 10^{-6}$, $s = 3.86$. For intermediate values of q (before the transition is complete) the statistical mechanical calculation gives $\tau_B = \tau_Z = -0.4$ deg per base pair. The transition profiles calculated at $T = 353$ K are virtually identical to those shown here. These results agree well with those from the thermodynamic analysis.

Theoretical analysis of transitions between B- and Z-conformations in torsionally stressed DNA

Craig J. Benham

Department of Mathematics, University of Kentucky, Lexington, Kentucky 40506

Recently, a new structure called Z-DNA has been proposed for alternating poly(dG-dC)·poly(dG-dC) sequences based on crystallographic analysis of the hexanucleotide¹. The Z-form is a left-handed double helix containing 12 base pairs per turn. In contrast, the Watson-Crick B-form helix is thought to have 10.4 pairs per turn of right-handed helix^{2,3,8}. A cooperative, salt-induced conformational transition has been observed in poly(dG-dC)·poly(dG-dC)⁴, which has been interpreted as

by $q < -bNA/4\pi^2 C_B$ before stable regions of Z-structure occur. When q satisfies this condition, unique non-zero values of n and τ_Z exist which minimize the conformational free energy⁵. These are

$$n = -qA - (bNA^2/4\pi^2 C_B) \quad (3)$$

$$\tau_B = \tau_Z = -bA/2\pi C_B \quad (4)$$

where $0 < n < N_Z$. (If the segment is sufficiently underwound that $n = N_Z$, then $\tau_Z = \tau_B$ is determined from equation (1). To illustrate these results we use values $C_B = 8.5 \times 10^{-12}$ erg base pair rad⁻² and $b = 0.8$ kcal mol⁻¹ from the literature^{4,7}. Then a segment must be underwound by 0.33 deg per base pair before the onset of transition to structures containing stable Z-form regions. When deformed beyond this threshold, enough susceptible base pairs switch to Z-structure for the twist rates to be maintained at $\tau_B = \tau_Z = -0.33$ deg per base pair. Only when the segment is so underwound that all susceptible sequences are in the Z-form do τ_B and τ_Z decrease below -0.33 deg per base pair.

To augment the above treatment we apply the statistical mechanical theory of two state transitions in torsionally stressed DNA⁶ to the present problem. This renders computable the transition probability profiles of specific base sequences, which more accurately reflect the fluctuational character of these transitions.

The partition function for this transition is⁶

$$Z = \sum_{n=0}^{N_Z} \left[I_n \sum_{\sigma} (s^n \sigma^\gamma) \right] \quad (5)$$

where r_n enumerates all states containing n Z-form base pairs, s is the equilibrium constant for a susceptible base pair, σ is the cooperativity factor and γ is the number of beginnings of sequences of base pairs in the Z-form. Also,

$$I_0 = \exp(-2\pi^2 q^2 C_B \beta / N), \quad (6a)$$

$$I_n = \left(\frac{2\pi(N-n)}{Nn C_B \beta} \right)^{1/2} \exp \left(\frac{-2\pi^2 C_B \beta (q + n/A)^2}{N} \right), \quad (6b)$$

$$0 < n \leq N_Z$$

From this partition function one can determine transition profiles, the expected value of τ_B and other equilibrium properties of the system as described in ref. 6. Figure 1 shows the results of a sample calculation carried out on a sequence of 80 base pairs, of which 8 were susceptible to the transition. Note that because the enthalpy of the B-Z transformation is zero⁴, this two-state transition is not thermal in character, but rather depends primarily on the imposed torsional deformation q .

We have presented two theoretical analyses of transitions between B-form and Z-form in torsionally stressed DNA. In this initial formulation the possible role of local denaturation (perhaps necessary at the junction between B-form and Z-form regions, for example) has not been considered. A three-state treatment of this problem will be presented elsewhere. Despite lack of precise knowledge of several important parameters (torsional stiffness C_Z , cooperativity factor σ), the present analysis predicts that B-Z transitions could occur in response to the torsional deformations consequent on (negative) DNA superhelicity. In principle, it is possible that similar transitions also occur between other conformational states of the DNA. It has been suggested that stress-induced conformational transitions at defined sequences may serve diverse regulatory functions by altering the accessibility of the bases⁵.

Metarhodopsin I/metarhodopsin II transition triggers light-induced change in calcium binding at rod disk membranes

U. B. Kaupp, P. P. M. Schnetkamp & W. Junge

Universität Osnabrück, Schwerpunkt Biophysik, Postfach 4469, D-4500 Osnabrück, FRG

The hypothesis of Yoshikami and Hagins¹ that calcium ions act as diffusible transmitter molecules between the photochemistry of rhodopsin and the subsequent electrical events at the outer plasma membrane of rods initiated many investigations on light-stimulated calcium release in vertebrate photoreceptor cells (see refs 2, 3). Although it now seems firmly established that light has some effect on the redistribution of calcium in various disk preparations^{2,4,5}, reconstituted systems^{6,7} and intact rod outer segments^{3,8}, the physiological significance remained unclear. We previously reported a rapid, light-triggered calcium release from binding sites at the disk membrane in the presence of calcium ionophore A23187 (refs 3, 8). However, there is no evidence for rapid calcium release into the cytosol in the absence of ionophore. On fragmentation of intact rod outer segments, calcium release due to a light-regulated change of calcium binding appeared almost completely abolished^{3,8}. We describe here experiments with sonicated rod outer segments in which the previously observed loss of the calcium release capacity has been prevented. Calcium release in sonicated disks in the presence of A23187 kinetically follows the metarhodopsin I/metarhodopsin II transition ($\tau_{1/2} = 10$ ms, activation energy $E_A = 34$ kcal mol⁻¹), suggesting that calcium release is triggered by this photochemical transition.

Intact cattle rod outer segments have been isolated according to Schnetkamp *et al.*⁹. Disk vesicles were obtained by sonication of intact rod outer segments in a medium containing sucrose, 600 mM; Ficoll 400, 5% v/v; HEPES, pH 7.0 2 mM, for 30 s at 40 W. The suspension also contained about 3–4 μ M of free calcium introduced by the stock suspension of rod outer segments.

Inhibition of calcium release can be prevented by keeping the ionic strength of the final suspension medium below 5 mM of 1:1 electrolyte, irrespective of the fragmentation procedure (lysis or sonication) and the fragmentation conditions (for example, high or low ionic strength). We conclude that the apparent abolition of the release capacity in fragmented rods is caused by regulation of calcium binding by cations other than calcium which had no access to the site of calcium release in both rods with a leaky and an intact plasma membrane.

Rapid calcium release was followed by means of kinetic flash spectrophotometry with the calcium indicator arsenazo (III). Details on working with arsenazo (III) and the principle and instrumentation of flash spectrophotometry have been reviewed elsewhere^{3,10}. In a suspension of sonicated disks buffered with only 2 mM HEPES at pH 6.8, pH measurements with the pH indicators bromocresol purple and Mg-arsenazo (III) (ref. 11) demonstrated that the total proton buffering capacity (membranes and HEPES) is sufficient to suppress light-induced pH changes which might give rise to pH-induced absorption changes of arsenazo (III) (not shown). To obtain kinetically undistorted recordings of calcium release in sonicated material at higher time resolution (Fig. 1a), signals due to the intrinsic photochemistry of arsenazo (III) (ref. 3) have been recorded separately and subtracted from the superimposed calcium-indicating signals by means of a signal averager with a data reduction program (Nicolet 1072). The signal-to-noise ratio was improved by sampling of signals over several repetitions from one cuvette.

Received 25 February; accepted 19 May 1980.

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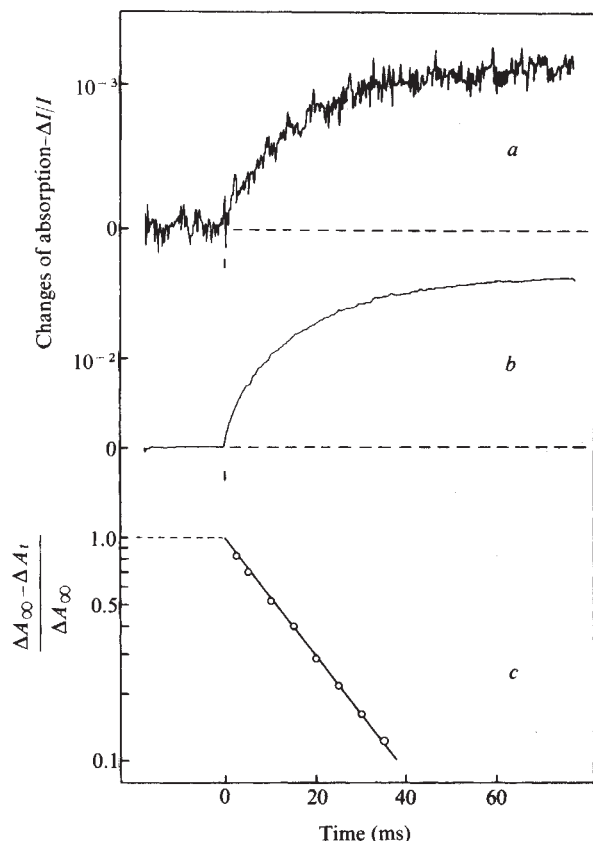


Fig. 1 Time course of the light-induced absorption changes at: *a*, 655 nm and *b*, 382 nm after excitation of sonicated rod outer segments by a flash at $t=0$ in the presence of arsenazo (III) and A23187. Signals were obtained by averaging from one sample over 16 repetitions for *a* and four repetitions for *b* at a frequency of 0.5 Hz. The optical bandwidth of the measuring beam was 16 nm, the electrical integration time 200 μ s. Further conditions as in Fig. 2. *c*, Semilogarithmic plot of the calcium-indicating absorption change from part *a*.

The half-rise time of flash-induced calcium release in the presence of equal amounts of A23187 (10 μ M) depends on the structural integrity of rod outer segments (Fig. 2). In intact rod outer segments, calcium release occurs more slowly ($\tau_{1/2} = 300$ ms) than in sonicated disk vesicles ($\tau_{1/2} = 10$ ms). Unlike its kinetics, the stoichiometry of calcium released/rhodopsin bleached is comparable in both preparations (~ 0.4 calcium/rhodopsin*). Light-triggered calcium release in sonicated material is depicted in Fig. 1*a* at a higher time resolution. A semilogarithmic plot of the absorption changes from Fig. 1*a* reveals that the time course of calcium release can be described by a single first order reaction (Fig. 1*c*). Concomitantly in intact rod outer segments and sonicated suspensions the metarhodopsin I/metarhodopsin II transition has been recorded at 382 nm. A half-rise time of about 10 ms at 20 °C and at pH 7 was observed for both preparations. The time course of this photochemical transition is shown for sonicated disk vesicles in Fig. 1*b*. From inspection of Fig. 1 it is evident that the time course of rapid calcium release in sonicated rods in these conditions closely parallels that of the metarhodopsin I/metarhodopsin II transition.

To determine the energy of activation for both processes, the temperature dependence of the respective rate constants has been investigated in similar conditions (Fig. 3). Rate constants have been calculated from overall half-rise times according to $1/\tau_{1/2} = k(\ln 2)^{-1}$. The metarhodopsin I/metarhodopsin II transition exhibits pH- and temperature-dependent biphasic kinetic behaviour (ref. 12 and our unpublished observation) and therefore two ways of analysing the data have been adopted.

First, the overall rate constants of the metarhodopsin I/metarhodopsin II transition are shown in Fig. 3 (closed circles). They match reasonably well with the calcium release data (triangles). If only the rate constant of the slower kinetic component is considered (Fig. 3, open circles) the correspondence, especially for higher temperatures, is even better. This is due to the temperature dependence of the relative amplitudes of the two kinetic phases of the metarhodopsin I/metarhodopsin II transition which favours the rapid component at higher temperatures¹². Notwithstanding this subtlety of a biphasic kinetic behaviour of the metarhodopsin I/metarhodopsin II transition, it is obvious from Fig. 3 that both reactions have a similar activation energy of about 34 kcal mol⁻¹.

The similarity of activation energies and time constants of both reactions strongly suggests that the calcium release system is intimately coupled to the metarhodopsin I/metarhodopsin II transition. In agreement with this notion is the linear relationship between metarhodopsin II formation and the amount of calcium release reported earlier^{3,8}.

The rapid rise time of the calcium-indicating absorption changes in sonicated vesicles probably reflects the actual time required for the trigger step to occur. The diffusion time of calcium ions from binding sites through the suspending medium to an indicator molecule¹³ as well as the relaxation time of complex formation between arsenazo (III) and calcium¹⁴ are too short to contribute significantly to the time course of the calcium-monitoring absorption changes. The mechanism underlying the slower kinetics in intact rod outer segments probably involves an additional reaction step and will be considered elsewhere¹⁵, but from the present study it is obvious that in the presence of 10 μ M A23187 the kinetics of calcium release are not rate limited by the ionophore.

For the actual release step two distinct mechanisms may be considered. In the first model, calcium is bound directly to rhodopsin and released during the metarhodopsin I/metarhodopsin II transition due to a change of the electrostatic interaction between calcium and its coordination groups at the rhodopsin molecule. This change could involve spatial separation of chelating groups accompanying conformational changes

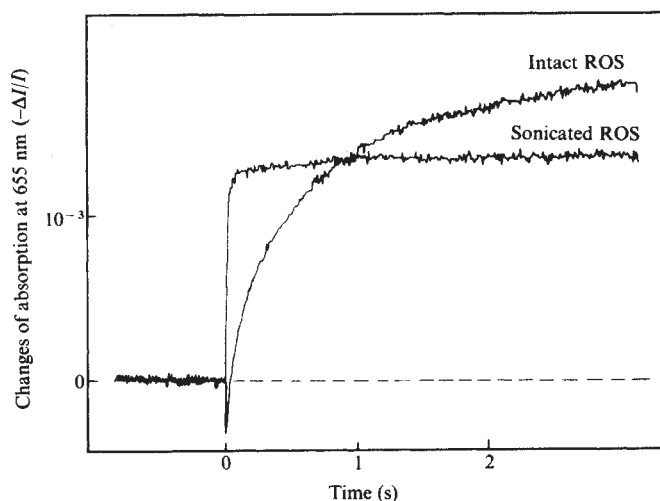


Fig. 2 Time course of the light-induced absorption changes at 655 nm after excitation of intact and sonicated rod outer segments (ROS) by a flash at $t=0$ in the presence of arsenazo (III) and ionophore A23187. Suspension medium contained: sucrose, 600 mM; HEPES, 2 mM; Tris, 0.5 mM at pH 7.0; arsenazo (III), 30 μ M; A23187, 10 μ M; free calcium concentration 3–4 μ M and rhodopsin, 3.5 μ M. Fraction of rhodopsin bleached/flash about 4%. Cuvette path length 10 mm. Temperature, 20 °C. Excitation wavelength, 530 nm; half duration of excitation flash 10 ns. Signals were obtained by averaging from one sample over four repetitions at a frequency of 0.1 Hz.

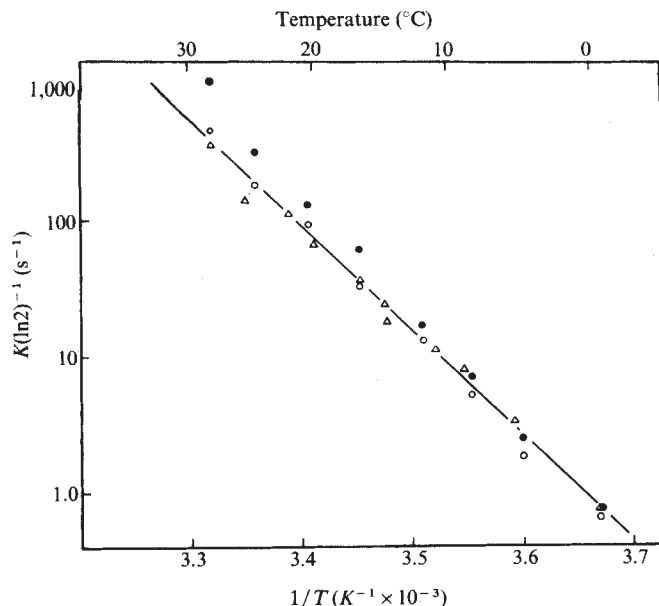


Fig. 3 Arrhenius plot of the temperature dependence of the calcium release (Δ), the metarhodopsin I/metarhodopsin II transition ($\bullet$), and the slow component of the metarhodopsin I/metarhodopsin II transition ($\circ$) in sonicated rod outer segments. Further conditions as in Fig. 2.

within rhodopsin. In the second model, a reorientation of molecular dipoles within the rhodopsin molecule or rapid proton uptake¹⁶ could decrease an interfacial potential at the disk membrane. According to $K_D = K_D^0 \exp(-2e\phi/kT)$ the apparent binding constant K_D of calcium binding sites (not necessarily on rhodopsin) is dependent on the surface potential ϕ (K_D^0 = binding constant in the absence of any interfacial potential). Consequently any change of an interfacial potential will result in a redistribution of calcium bound to the membrane.

It is difficult to decide between the models outlined above. The close correlation between calcium release and the metarhodopsin I/metarhodopsin II transition could be interpreted in favour of the first model. However, the fact that rapid calcium release is completely abolished when rod outer segments are dissolved in either nonylglucose (gift of Dr W. J. de Grip, Nijmegen) or Triton X-100 (not shown) would be difficult to explain if calcium were directly bound to rhodopsin. A more detailed account of the interrelationship between calcium release, rapid proton uptake and interfacial potentials will be published elsewhere¹⁵.

This work was financially supported by the Deutsche Forschungsgemeinschaft.

Received 21 February; accepted 19 May 1980.

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A novel conformation of valinomycin in its barium complex

S. Devarajan, C. M. K. Nair, K. R. K. Easwaran & M. Vijayan

Molecular Biophysics Unit, Indian Institute of Science, Bangalore-560 012, India

Knowledge of the molecular mechanisms involved in ionophore-mediated cation transport would be valuable for understanding many essential functions of biological membranes^{1–3}. Cations are transported in several stages, such as formation of the ionophore-cation complex, diffusion across the cell membrane and subsequent release of the cation. Several conformational rearrangements are involved in this process, and so a detailed understanding of all the conformational possibilities of the ionophore seems to be essential for elucidating the molecular mechanism of ion transport. We are carrying out spectroscopic and crystallographic studies to explore the possible conformational stages of ionophores by complexing them, in different solvents, with cations of various sizes and charges. We report here a novel conformation of the ionophore valinomycin in its barium complex. It can be described as an extended depsipeptide chain, without internal hydrogen bonds, wound in the form of an ellipse with the two barium ions located at the foci.

Spectroscopic studies in solution (for example, circular dichroism (CD) spectra shown in Fig. 1) carried out by us indicated that the conformation of valinomycin in its barium complex was different from those in uncomplexed valinomycin and its complex with monovalent cations. Barium perchlorate and barium thiocyanate were used to investigate complexation

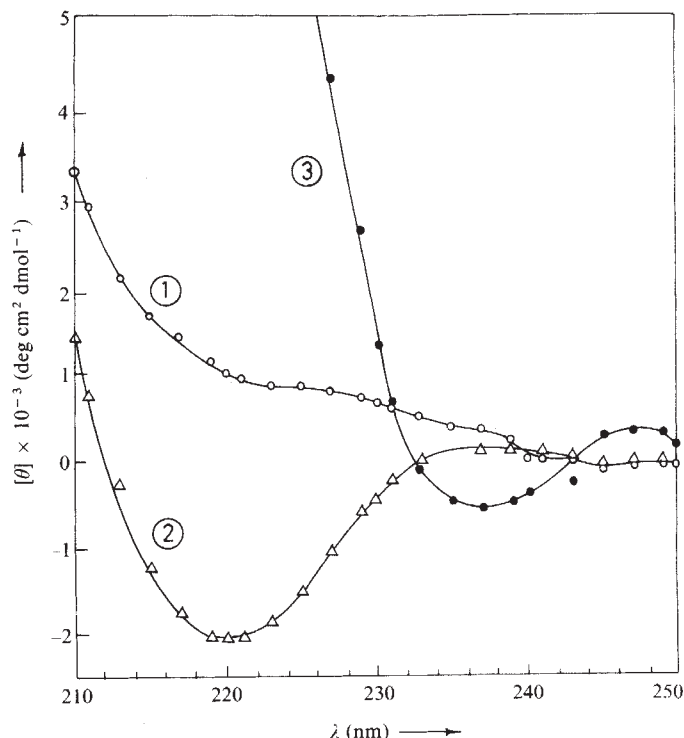


Fig. 1 CD spectra in acetonitrile of free valinomycin ($\circ$), its 1:2 barium perchlorate complex (Δ) and its 1:1 potassium perchlorate complex ($\bullet$). The concentration of valinomycin was 29 mM ($\circ$, Δ) and 9 mM ($\bullet$). In the potassium perchlorate complex the molar ellipticity, $[\theta]$, continuously increases with decrease in λ with a $[\theta]$ value of 25,000 deg cm² per dmol at $\lambda = 210$ nm.

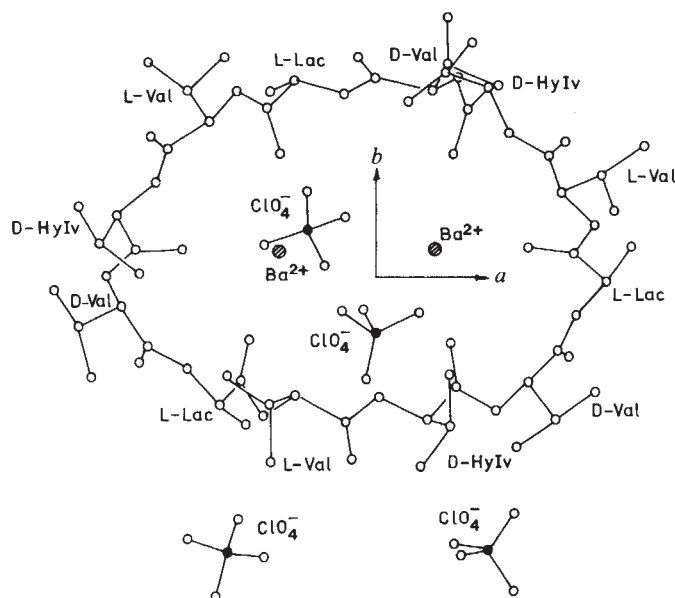


Fig. 2 The location of the valinomycin molecule, the barium ions and the perchlorate groups as viewed along the *c* axis in the crystals of a 1:2 valinomycin-barium perchlorate complex.

of valinomycin with Ba^{2+} . CD data on the titration of barium perchlorate with the ionophore in acetonitrile indicated valinomycin- Ba^{2+} complexes with 2:1, 1:1 and 1:2 stoichiometries, although the conformation of the molecule stabilized only at high salt concentrations (S.D. and K.R.K.E., in preparation). CD could not be used with barium thiocyanate because of the high UV absorption of the thiocyanate group. However, proton NMR indicated a stable 1:2 valinomycin-barium thiocyanate complex, although the titration curve based on NMR data for the thiocyanate salt was different from that for the perchlorate salt (S.D. *et al.*, in preparation). Thus, it seemed that valinomycin could form 1:2 complexes with barium perchlorate as well as barium thiocyanate and we decided to attempt X-ray crystallographic studies on these complexes.

Complexes (1:2) of valinomycin with barium perchlorate and barium thiocyanate were crystallized by slow evaporation of slightly aqueous acetonitrile solutions containing the respective components in molar proportions. The crystals were mounted in thin-walled glass capillaries together with the solvent. X-ray diffraction photographs showed that the valinomycin-barium perchlorate complex crystallizes in the orthorhombic space group $P2_12_12_1$ with $a = 28.304 \text{ \AA}$, $b = 16.938 \text{ \AA}$, $c = 19.543 \text{ \AA}$ and $z = 4$, whereas the thiocyanate complex crystallizes in the monoclinic space group $C2$ with $a = 29.48 \text{ \AA}$, $b = 16.49 \text{ \AA}$, $c = 19.92 \text{ \AA}$, $\beta = 111.4^\circ$ and $z = 4$. X-ray intensity data from both the crystals were collected on a computer controlled four-circle CAD-4 diffractometer using graphite monochromatized $\text{MoK}\alpha$ radiation to a maximum Bragg angle of 23° . The structure of the barium perchlorate complex was subsequently determined by the heavy atom method and refined by the block-diagonal structure factor least squares technique to an R value of 0.13 for 3,504 observed reflections ($I > 2\sigma(I)$). So far, the two barium ions and all the non-hydrogen atoms belonging to the valinomycin molecule, the four perchlorate groups and four water molecules have been located.

The location of the barium ions, the valinomycin molecule and the perchlorate ions in the crystal structure is shown in Fig. 2. The main-chain dihedral angles⁴ in the valinomycin molecule correspond approximately to those for an extended chain. The molecule can be described as an extended depsipeptide chain, with no internal hydrogen bond, wound in the form of an ellipse with the barium ions located at the foci. Three consecutive amide carbonyl groups coordinate to one of the barium ions whereas the remaining three consecutive amide carbonyl groups coordinate to the other barium ion. The coordination of the

cation is completed by perchlorate and water oxygens. The conformation of valinomycin observed in the structure is totally different from those found in the crystal structures of uncomplexed valinomycin^{5,6} and its potassium complex⁷. It is also different from the conformations unambiguously characterized so far in solution studies¹⁻³.

We thank the University Grants Commission and the Department of Science and Technology, India, for financial assistance, and Dr A. M. Shaik for help with data collection. The atomic coordinates have been deposited in the Crystallographic Data Bank, University Chemical Laboratory, Lensfield Road, Cambridge, UK.

Received 3 March; accepted 5 June 1980.

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Circular polarization observed in bioluminescence

Hans Wynberg*, E. W. Meijer*, J. C. Hummelen*, H. P. J. M. Dekkers†, P. H. Schippers† & A. D. Carlson‡

* Department of Organic Chemistry, University of Groningen, The Netherlands

† Department of Theoretical Organic Chemistry, University of Leiden, The Netherlands

‡ Department of Neurobiology & Behavior, State University of New York at Stony Brook, Stony Brook, New York 11790

While investigating circular polarization in luminescence^{1,2}, and having found it in chemiluminescence^{3,4}, we have studied bioluminescence because it is such a widespread and dramatic natural phenomenon^{5,6}. We report here that left and right lanterns of live larvae of the fireflies, *Photuris lucifrescens* and *Photuris versicolor*, emit circularly polarized light of opposite sense.

Firefly larvae (Fig. 1) were gathered in the US and sent by airmail to The Netherlands. They were alive up to and during most of the experiments. In our first attempts to detect polarization of luminescence, we measured the total light emission from both lanterns of the larvae. The results were puzzling and disappointing; vanishingly small circular polarization was observed, with only a few exceptions. To our surprise, we noted—fortuitously—that measurements of the light emission from the left or right lanterns separately gave more constant and encouraging results. During the latter experiments one important fact was established. By shifting and rotating the lanterns through many angles and positions with respect to the optical axis of the apparatus, we found that orientational artefacts could not be the origin of the circular polarization subsequently measured. Whereas in these early experiments we used excised lanterns rigidly mounted in a cell, in the later experiments we preferred to attach entire larvae with fine wire to a holder. The holder was positioned in front of a diaphragm so that one lantern only was fixed onto the optical axis of the apparatus. Continuous bioluminescence, lasting from several minutes to an hour, was achieved by feeding the larvae with a concentrated solution of racemic amphetamine hydrochloride in water. Luminescence usually began 5–30 min after the addition of amphetamine. Measurements were then made immediately.

The circular polarization of luminescence was measured as the anisotropy factor or g_{lum} factor $(I_L - I_R)/\frac{1}{2}(I_L + I_R)$. The difference in the numbers of left and right circularly polarized

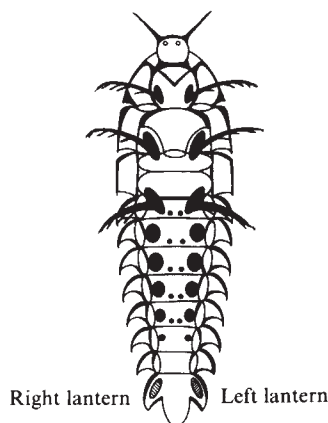


Fig. 1 Sketch of the firefly larva (ventral view). The shaded spots at the bottom represent the lanterns.

photons emitted, $I_L - I_R$, was modulated at a frequency of 50 kHz and detected as alternating photocurrent; the average luminescence $\frac{1}{2}(I_L + I_R)$ was measured as direct photocurrent^{1,2,7-9}. Measurements of circular polarization of bioluminescence (CPBL) of 16 firefly larvae lanterns are shown in Fig. 2, revealing that the left and right lanterns emit polarized light of opposite sense. Within each group there is a considerable variation in the total light emission and values of g for the individual lanterns. In these experiments, the construction of the sample holder prevented a detailed investigation of orientational effects. However, on the basis of results of earlier experiments, we believe that neither position effects nor those due to linear polarization could have decisively affected our result.

What is the origin of the CPBL, and what is the origin of the difference in handedness shown by the left and right lanterns? According to the accepted mechanism of firefly bioluminescence mediated by luciferin, an achiral emitting state, III^* (see Fig. 3), is involved^{5,10-13}. This achiral excited state, by itself, offers no possibility of explaining the CPBL. It may be argued however, that the true excited state consists of a complex between the achiral III^* and the enzyme luciferase. Such a chiral excited state complex should, in principle, be capable of emitting circularly

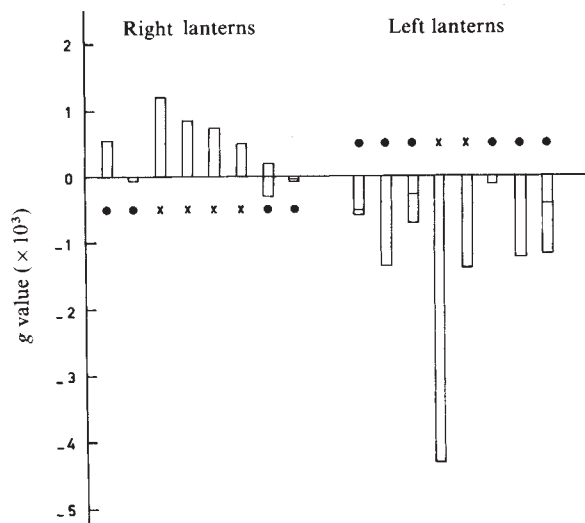


Fig. 2 Results of measurements of circular polarization of bioluminescence of firefly larvae lanterns with sufficiently high emission intensity. The height of each bar represents the g value for an individual lantern of *Photuris lucifescens* (X) or *Photuris versicolor* (●). Superimposed bars refer to the effect of orientational changes (see text). The circular polarization is measured at the peak of the emission band at a wavelength of 540 nm using a spectral bandwidth of 40 nm. The r.m.s. noise level corresponds with $g \leq 5 \times 10^{-4}$.

polarized light^{3,4}. A second explanation would invoke a circularly dichroic medium capable of partially absorbing the emitted light. Neither of these two modes can explain why the left and right lanterns emit polarized light of opposite sense, unless enantiomeric enzymes, active sites or membranes are proposed. In the absence of any evidence for enantiomeric structures, we are forced to conclude that the CPBL of opposite sense must, at least in part, be based on a macroscopic phenomenon rather than on molecular chirality.

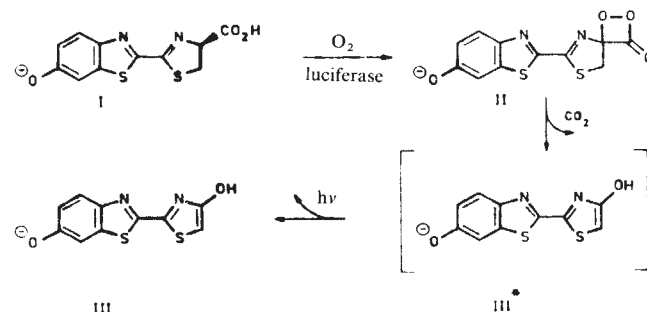


Fig. 3 Schematic mechanism of luciferin/luciferase bioluminescence. Oxygenation of luciferin (I) yields the intermediate II which in turn releases CO_2 , resulting in the production of III (or, by H^+ -abstraction, the enol dianion) in an electronically excited state. This state emits a quantum of light as it goes to its electronic ground state.

We propose the following hypothesis. The light from a bioluminescent emitter may be (partially) plane polarized due to anisotropy of an absorbing medium or, more likely, by inhomogeneous formation of excited states due to local molecular organization within a photocyte¹⁴. This plane polarized light will become elliptically polarized when it passes through a linearly birefringent medium. Orientated biopolymers can serve as such a medium. On a macroscopic scale the larvae, like many living organisms, have a symmetry plane dividing the lanterns. In this sense the lanterns are enantiomeric, even though their constituent molecules are of the same chirality; they may be viewed as macroscopic meso-structures. It is reasonable to assume that this macroscopic mirror image relationship holds on the level of the membrane structure and orientation of the emitters. This will result in circularly polarized light of opposite sense. The emitters from one lantern emit partially plane polarized light making angle $+X^\circ$ with the fast axis of its linear birefringent medium. The result is ellipticity of one sense ($+Y$). The mirror image relationship of the emitters in the other lantern results in partially plane polarized light with angle $-X^\circ$ with respect to the fast axis of its linear birefringent medium. This must result in ellipticity of opposite sense ($-Y$). We may expect, then, that the meso-orientated lanterns in any bioluminescent organism will emit circularly polarized light of opposite handedness, provided the chirality on the molecular level does not exceed the effect. The results described above suggest experiments dealing with the linear polarization of firefly larvae bioluminescence. Such experiments are planned.

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BOOK REVIEWS

Fuel for the 'back-end' debate

Ian Smart

THE 'back end' of the commercial nuclear fuel cycle, covering what happens to fuel material after its irradiation and withdrawal from power reactors, has had more attention and words lavished on it since 1976 than, arguably, it deserves. It contributes a trivial proportion to the cost of nuclear electricity. It offers more technical choices, and fewer imperatives, than earlier stages in the cycle. And, while no one doubts that plutonium from spent power reactor fuel can be used in nuclear explosives, the danger of that particular route to nuclear proliferation, in relation to others, has frequently been exaggerated. Yet the floods of emotion and typescript continue to rise around the fuel cycle's back end: around nuclear waste management, around reprocessing and the recycling of its products, and around the handling of spent fuel itself.

Reasons for this almost obsessive concern are obvious: the prospect of a so-called 'plutonium economy', based on exploiting back-end opportunities, is genuinely arresting. On the one hand, the consequential introduction of commercial fast breeder reactors is seen by some as a necessary graduation from nuclear infancy, but by others as a leap into economically uncharted and technically dangerous obscurity. On the other hand the issue of nuclear weapons proliferation raised by widespread commercial separation of plutonium is quantitatively, even if not qualitatively, new. And, to cap it all, any discussion of the back end, with or without reprocessing, touches the problem of nuclear waste, management of which provokes more environmental fears — together with more complacency, superstition and reciprocal condescension — than any other topic in the nuclear debate. One may regret that so much time is spent in writing or reading about the back end, at the risk of ignoring what may be more pressing difficulties in the rational and safe development of nuclear power, but one cannot be surprised.

The pathetically familiar polarization of the back-end debate is well illustrated by conflicting images of spent reactor fuel itself: on one side the image of spent fuel as a 'plutonium mine' of immediately valuable fissile material, to be extracted and exploited for good or ill, on the other the image of it as merely one form of nuclear waste, to be buried away out of sight and reach as quickly as possible. Both

Nuclear Nonproliferation: The Spent Fuel Problem. Edited by Frederick C. Williams and David A. Deese. Pp.221. (Pergamon: New York and Oxford, 1980.) \$30, £15.

views are caricatures, justifiable only by ignoring the time factor. In the main, spent fuel is currently a nuisance, encumbering storage ponds at reactor sites and imposing additional costs on utilities. Yet the reactors concerned have extracted less than 1% of the energy potentially available in the uranium originally mined. The rest remains locked up in the spent fuel, as plutonium and depleted uranium, or in the tails at enrichment plants, to be extracted only if and when fast breeder reactors are commercially feasible and widely available. That day may not come, and spent fuel may remain a radioactive nuisance. Meanwhile, however, to treat it as nothing but waste would be like refining crude oil merely to extract bitumen for roads, and then setting fire to all that remained because the internal combustion engine was not yet ready for commercial service. Today's burden may be tomorrow's scarce resource.

One merit of this book is that it studiously avoids caricaturing spent fuel as either immediate gold or permanent dross. Although it starts from the premise, espoused by the Carter administration, that commercial reprocessing is currently uneconomic and politically undesirable, it acknowledges that circumstances may change, and that the ways in which spent fuel is handled today must not preclude using its plutonium content in the future. From there, it moves to the judgement that, to ease pressure on storage at reactors while also preventing an allegedly dangerous increase in the volume and distribution of reprocessed plutonium, more ambitious provision must be made for away-from-reactor (AFR) storage, which could usefully be under some kind of international control. The task it then sets itself is to explore the manner in which an international spent fuel storage arrangement might be set up in different regions of the world.

So far, so good. Whether or not too much time has recently been spent in agonizing over the back end, nothing like enough careful thought has yet been given to how, in the real world, international or multinational arrangements might reduce the fears of proliferation, environmental

damage or public rejection which surround various stages of the fuel cycle. It is encouraging, therefore, to find that "a working group of fifteen scholars and professionals, representing a diversity of academic levels, professions, experience, and nationalities, met biweekly over the course of a year" at Harvard, and that here we have 11 of their papers, dealing not only with politics, technology and economics at a general level but also with five major world regions. Marvin Miller shows that, although more development is needed, there are technical methods of storing spent oxide fuel safely (and retrievably) for at least 25 years. Boyce Greer and Mark Dalzell outline an ingenious economic model which suggests that all except the largest reactor-operating countries might well save a lot of money during that period by sending their spent fuel for storage in large international facilities, even at a considerable distance. The editors, David Deese and Frederick Williams, provide an introductory discussion of the political options available and of the political criteria which are likely to determine the location of any international spent fuel store, and reach the sensible conclusion that the right approach to building an international regime is a gradual and incremental one, serving energy as well as non-proliferation concerns and eschewing dramatic or, above all, unilateral, policy excursions. And Tariq Osman Hyder adds a crucial warning that no such regime will be created unless developing as well as developed countries are convinced in advance of its equity. A foundation of realism and feasibility seems to have been laid.

Thereafter, unfortunately, everything slides downhill. The papers on political prospects in specific regions, clearly drafted as essays for a biweekly seminar, are altogether slighter contributions, uneven in technique and quality and sometimes uncertain on fact. All are ultimately pessimistic. Melvyn Nathanson, while offering the eccentric thought that the USSR might store spent fuel from outside Comecon, regards internationally controlled storage arrangements as irrelevant to Eastern Europe. Onkar Marwah sees none but the remotest prospect of Indian Ocean countries subscribing to such an arrangement, and then only if a regional store on a remote French or Australian island accepted

nothing but fuel from a few fully safeguarded plants. Victoria Johnson and Carlos Astiz are driven back to suggesting Paraguay or Uruguay as the only possible hosts for a Latin American store. Richard Broinowski is hardly more optimistic about Asia and the Middle East, and Robert Gallucci even less encouraging, if that is possible, about Western Europe. At the end, therefore, the editors, concluding sadly that there is little chance of rational economic arguments overriding "the centrifugal forces of international politics", leave one asking whether the dinner was worth the detour.

It may be because their surveys of regional politics so depressed them that the editors and authors found no reason even to consider some of the most obtrusive problems in working towards international or multinational fuel cycle arrangements. There is no discussion here of the criteria which should govern the release of spent

fuel from international custody. There is almost nothing about institutional form or organization: how rights, responsibilities, costs or authority might be pooled, distributed or devolved. These are glaring omissions, which inevitably detract from the book's value. They might nevertheless be justified if the political prospects were as impenetrably gloomy as here indicated. The hard fact, however, is that the political analyses in the volume often verge on the simplistic, and are forced, moreover, towards pessimism by questionable underlying assumptions: that international arrangements for spent fuel storage must, for example, be addressed in isolation from other fuel cycle functions, or that membership of any multinational fuel cycle institution must be drawn from a single geographical region. Above all, those responsible for the study have steered so far away from the Scylla of believing that political obstacles will vanish in the

face of an intellectually coherent scenario that they have fallen victim to the Charybdis of equating the politically difficult with the palpably impossible.

The authors are to be praised for venturing into turbulent waters which it may be politically essential for governments to explore thoroughly during the next few years, and for setting up some preliminary aids to navigation. At the end of the day, however, their courage seems to have failed, because they have judged the icebergs of politics to be immutable. It is not necessary, and it may not be wise, to assume that national policies must be as unalterable, or diplomacy always as impotent, as their conclusions would suggest. □

Ian Smart is an independent adviser on international energy affairs, and was Chairman of the International Consultative Group on Nuclear Energy (1977-80).

Learning from the Arctic peoples

John Ebling

The Human Biology of Circumpolar Populations. International Biological Programme 21. Edited by F.A. Milan. Pp.367. (Cambridge University Press: Cambridge, 1980.) £35, \$75.

THE Arctic regions were the last major environment to be conquered by *Homo sapiens*. The radiation of human beings into colder climes northwards and southwards from the tropics probably started about half a million years ago and extended into western and central Europe under conditions of great cold during the Pleistocene. At the end of the Old Stone Age, the use of clothing enabled the human hunters to follow the retreating ice of the Third Würm Glaciation into the Eurasiatic land mass and finally to move into the New World. What adaptations were involved? In general, the physiological differences between modern circumpolar peoples and those more favoured climatically are not striking. They may, perhaps, show an earlier onset of cold vasodilation and have uniformly warmer fingers, but their eyes differ little, even though they are exposed to continuous low temperatures, blowing snow in winter and high ultraviolet radiation in spring. In the far Arctic, people survive mainly on protein, without any obvious source of essential vitamins, and generate necessary glucose from amino acids. The polyunsaturated fats from seal and whale oils keep plasma cholesterol and heart disease at enviably low levels.

The Human Biology of Circumpolar

Populations is an admirably edited source book with contributions from Canada, the USA, Denmark, Norway, Finland, France and the USSR. The chapters deal in turn with the demography, genetics, cranio-facial features, ophthalmology, anthropometry, nutrition, physiology and behaviour of the 16 indigenous Arctic peoples, ranging from the Lapps of Scandinavia to the Eskimos of Greenland and including the lesser known Siberian groups and the Ainu.

The publication of such a book, and the

special studies of the International Biological Programme which went into its making, are timely. The circumpolar peoples are changing. Alcoholism, leading to accidents, violence and other social disorders, is on the increase. There is much that our society needs to learn from them before they are destroyed by contact with us. □

John Ebling is Professor of Zoology in the University of Sheffield, UK.

Ecological view of marine animal diseases

R.J. Roberts

Diseases of Marine Animals. Vol.1. Edited by O. Kinne. Pp. 466. (Wiley: Chichester, UK, and New York, 1980.) £25, \$73.15.

BECAUSE of the span of our knowledge, and the complexity of the subject, pathology books are nowadays generally multi-author volumes which deal with a single species (usually human beings) or even a single organ system. Comparative pathology texts are few and generally relate to specifically defined groups of economic significance such as domesticated animals or teleost fish. Only a brave, or naive, person would attempt to write a textbook covering the diseases of virtually the entire animal kingdom in so far as it inhabits the marine environment. Surprisingly this brave man is not Otto Kinne, whose name appears as editor on the cover of this first volume in a series of four, but one G.H.

Lauckner who has apparently been responsible for the entire content of all four volumes apart from the two very general introductory chapters. He appears to have received less than adequate credit for this mammoth task.

Dr Kinne is a distinguished marine ecologist and in the foreword to this first volume he gives his view that disease is primarily an ecological phenomenon. This is a very different attitude to that of the majority of comparative pathologists and, I suspect, of commercial aquaculturists and fishery biologists as well. It is true, however, that marine ecologists have too often focused exclusively on holistic ecosystem properties and food-web interrelations in ignorance of the impact of diseases on populations. It is a stated aim of this series to synthesize all of the information available to allow the ecological significance of diseases in the marine environment to be appreciated.

This first volume covers the lower invertebrates, ranging from the Protozoa to the Gastropoda. These are the groups covered by the smallest amount of published literature in comparative pathology, and thus for many of them the

scarcity of documentation is perforce out of all relation to their importance to the marine ecologist. This is no fault of Dr Lauckner's but it does impede the objective of helping biologists to give disease its appropriate place within the marine ecological model.

Since very few of the invertebrates covered in this volume are cultured, or even fished, the economic incentive for the detailed scientific study of their diseases has been absent. Most of the records of such diseases are one-off observations

rather than systematic studies and often even Dr Lauckner is unable to do more than detail the paucity of available information.

Although the book is well produced and beautifully printed, most pathologists (though not, I suspect, parasitologists) would jib at the extensive use of line drawings rather than photomicrographs in all but a very limited number of instances.

The gathering together of such a mass of information, often related only by its ecosystem, and its synthesis into a review

of this magnitude indicates great determination. As a source of information for scholars of a wide range of subjects it will be invaluable. I doubt, however, if it will substitute for a specific monograph on any of the groups covered. It is neither specialized enough nor complete. I do expect, though, that the complete series will find its place in any library attempting to cover the field of marine biology. □

R.J. Roberts is Director of the Institute of Aquaculture at the University of Stirling, UK.

An 'axiomatic' approach to quantum mechanics

J.L. Martin

Quantum Mechanics. By A. Böhm. Pp. 522. (Springer: Heidelberg and New York, 1979.) DM 58, \$31.90.

IN WRITING about quantum mechanics, where does an author begin? There are several possibilities. He may struggle through the historical development of the subject, with all its gropings and false starts — excellent in its place, but not in a standard text. He may begin with elementary physical examples, and using a judicious mixture of assertion, intuition, correspondence principle and Ehrenfest's theorem — uncluttered with too much mathematics — attempt to lead the reader into a 'feel' for the subject. Or he may adopt an 'axiomatic' approach: 'Here are the rules; where do they lead?'. The trouble with this last option is that the reader who is looking for enlightenment may have to journey for too long in the dark before the dawn of what it is all about.

This is an 'axiomatic' book, and I would hazard the opinion that it is not for the physicist — not even for the theoretical physicist — but for the mathematician who is already familiar with the basic structure of quantum mechanics. Moreover, the selection of topics obviously reflects the author's personal interests to a degree; thus I find it difficult to recommend the book as a text, as the coverage is so uneven. It may well be useful to anyone with an interest in the mathematical aspects of quantum mechanics, developed from a particularly algebraic point of view. (The very first mention of a physical system, p.10, runs: "The mathematical image of a physical system is an operator $\ast$ -algebra in a linear scalar-product space $\mathcal{H}$ ". This sets the tone of the book as a whole.) The reader who wishes to learn to use quantum mechanics as a tool will be disappointed; a glance at the problems at the end of each chapter will confirm this. Compare them, for example, with those in classics such as Schiff

(*Quantum Mechanics*. McGraw-Hill: New York, 1968).

In reading the book, I felt I was listening to the lectures. The presentation in parts was too discursive and rambling for my taste: after all, the reader can re-read the text if he needs to, and writing a book is a golden opportunity to tighten up a lecture style into something more terse and tidy, in which the wood can be seen in spite of the trees.

There are many small and usually

unimportant misprints which betray a lack of care at the proof-reading stage — "communication relations" (p.41); in the index under "Selection rules", "hydrogen bomb" should surely be "hydrogen atom". And by the way, the Argand diagram was not introduced to help us understand the scattering amplitude. Jean Robert Argand did his work in 1806! □

J.L. Martin is Reader in Physics at King's College, University of London, UK.

Controversial view of primate evolution

R. D. Martin

Evolutionary History of the Primates. By Frederick S. Szalay and Eric Delson. Pp.580. (Academic: New York and London, 1980.) \$48, £31.20.

THERE has been a great proliferation of research into the evolutionary radiation of the primates over the past 15 years. Re-analysis of the evidence has benefited from new palaeontological work, from a virtual explosion of new information on living primates (notably including biochemical data) and, above all, from reassessment of the methods used to infer phylogenetic relationships. As primate palaeontologists, Frederick Szalay and Eric Delson have both made significant contributions in the first of these areas, and *Evolutionary History of the Primates* is accordingly an authoritative and competent guide to the great array of recognized primate fossils.

A brief introduction includes, among other things, a handy chronological catalogue of known primate fossil sites and a simple outline of primate molar tooth morphology. The methods used for inferring evolutionary relationships, which owe much to Hennig, are touched upon; but this all-important aspect should have been discussed in full detail, especially since the authors themselves admit to a divergence of opinion. The bulk of the book consists of a systematic survey of primate groups, with notes on extant forms

and extensive, well-illustrated descriptions of the fossils. In this respect, the book will undoubtedly serve as a valuable reference source and its usefulness is enhanced by a selective bibliography and a glossary of terms. Numerous good-quality scaled photographs combine to make this the most comprehensive primate fossil guide available to date, and it is to be recommended to anyone with an interest in primate evolution.

A sharp distinction must be drawn, however, between the mere description of fossil primate material and its interpretation within a comprehensive evolutionary framework. A severe limitation of this book lies in the exclusion of virtually all information other than that derivable from teeth and bones. It is particularly surprising that there is barely a reference to the evolution of the primate brain, since endocasts are now available to document fundamental features in numerous fossil primate species. In their introduction the authors accept that the reliability of phylogenetic inferences increases with the scope of the data analysed, yet they ignore or even discount out-of-hand much relevant information (e.g. immunological data) derived from living primates. A proper synthesis of both palaeontological and neontological data must be achieved before maximal accuracy can be expected.

The rationale underlying the classificatory scheme used in the book is also inadequately explained. In places, new taxonomic groupings have been established as direct concomitants of the authors' personal hypotheses regarding evolutionary affinities, whereas elsewhere

this strict Hennigian approach has not been followed. Some innovations have much to commend them; others will generate little but confusion. It is probably a wise move to erect a separate suborder Plesiadapiformes to accommodate *Plesiadapis* and its apparent relatives, thus emphasizing the enormous morphological gulf between these early Tertiary forms and the "primates of modern aspect" (Simons). Indeed, it is by no means certain that the plesiadapiforms exhibit anything other than convergent dental and basicranial similarities to the more obvious primates.

On the other hand, there are several taxonomic changes which could well have been omitted without in the least detracting from the authors' provisional evolutionary hypotheses. Szalay and Delson do not combine all of the primates of modern aspect (their "euprimates") into a formal taxonomic category, despite abundant evidence that this is a monophyletic grouping, so it is difficult to see why other classificatory novelties were required. Two examples illustrate the problems. Firstly, in line with a few other authors, the lemur family Cheirogaleidae has been moved from the superfamily Lemuroidea (containing all the other Malagasy lemurs) to join the Lorisidae in the superfamily Lorioidea. Szalay's interpretation of certain morphological evidence, which is shared by some but has been disputed by others (and which is in direct conflict with available biochemical evidence), suggests a recent common ancestry for cheirogaleids and lorises. Whether or not this hypo-

thesis proves to be tenable in the long term, the immediate practical outcome of the taxonomic upheaval is that the term 'lorisoid', which has had a distinct and useful meaning for decades, has been rendered ambiguous. According to which classification one now follows, the word 'lorisoid' may or may not subsume the mouse lemurs and their relatives. Similarly, Szalay and Delson have reduced the marmoset and tamarin group from family level to the tribe Callitrichini and have lumped them together with *Callimico* and the Cebinae (*Cebus* + *Saimiri* + conjectured fossil relatives) into the family Cebidae. This drastic modification is based on Rosenberger's tenuous interpretation of minor dental and cranial features as "shared derived characters" linking marmosets and tamarins to cebines and is once again in conflict with immunological evidence. Henceforth, to the confusion of many involved in research upon marmosets and tamarins, the term 'cebid' might denote either 'all New World monkeys other than marmosets and tamarins' (this being the almost universally accepted meaning to date) or 'marmosets, tamarins, *Callimico* and cebines'.

Must we pay the price of such linguistic confusion as provisional hypotheses are generated in the pursuit of a more scientific understanding of primate evolutionary history? □

R. D. Martin is Reader in Physical Anthropology at University College, and Visiting Professor in Zoology at Birkbeck College, University of London, UK.

Conformation of saturated heterocycles

A.R. Katritzky

The Conformational Analysis of Heterocyclic Compounds. By Frank Riddell. Pp. 153. (Academic: London and New York, 1980.) £17, \$39.50.

THIS SMALL book will be of considerable help not only to organic and physical chemists, but also to workers in biochemistry and in other biological areas who need to understand something of the conformational analysis of saturated heterocycles. The first two chapters set the scene by summarizing the fundamental concepts on which conformational analysis is based, and then by picking out and emphasizing the differences which characterize the treatment of saturated heterocycles as compared with their carbocyclic analogues.

The remaining two-thirds of the book is organized into five chapters dealing with (1) 4- and 5-membered rings, (2)

6-membered oxygen-containing rings, (3) 6-membered nitrogen-containing rings, (4) 6-membered rings containing sulphur or phosphorus, and (5) 7-membered and larger rings. To treat this variety of topics within 150 pages, the author must of course be selective: he has rightly chosen to discuss the most important and fundamental ring systems, and thus little attention is paid to polycyclic derivatives. He has wisely decided to describe, clearly and in some detail, a smaller number of compounds rather than to attempt an encyclopaedic treatment which could have degenerated into mere lists.

The work has obviously been a labour of love; the proof-reading has been carried out excellently, the diagrams are well drawn and clear, and the book has an authoritative ring about it. Further, the subject has not been without its controversial features and on the whole the author deals fairly with these aspects. □

A.R. Katritzky is at present Professor of Organic Chemistry and Dean of the School of Chemical Sciences at the University of East Anglia, UK, but will be taking up a new appointment as Kenan Professor of Organic Chemistry at the University of Florida, Gainesville, in September 1980.

Soviet physics

A. J. Leggett

Soviet Scientific Reviews. Section A: Physics Reviews Vol.1. Edited by I.M. Khalatnikov. Pp.305. (Harwood: Chur, Switzerland, 1979.) Dfl.125, \$67.75.

THIS book is one of a new series which is intended to bring to English-speaking physicists some of the highlights of current research in the Soviet Union. This first volume contains six review articles which concentrate primarily on advances made by Soviet physicists in papers published in 1977, although reference is made to earlier, and contemporary non-Soviet, work as appropriate. The reviews fall conveniently into three groups of two papers each: Belavin (on instantons) and Zakharov and Manakov (on solitons) concentrate on mathematical developments in field theory, particularly the use of algebraic geometry, and make little if any reference to experiment; the papers by Volovik (superfluid ^3He) and Ivlev (nonequilibrium phenomena in superconductors) are largely theoretical but refer to relevant experiments from time to time; while those by Pokrovskii and Timofeev (exciton condensation in semiconductors) and Borovik-Romanov and co-workers (spin waves in antiferromagnets) are mainly experimental in content.

These reviews are definitely not of the type which could usefully be picked up by a non-specialist as an introduction to the subject in question. They will probably be most useful to those who, while not actively enough involved in a particular area of research to wish to spend a lot of time reading the original Soviet literature, are nevertheless close enough to it to want to keep up with important developments in some detail. For them, this book may play the role as regards Soviet physics that occasional lectures and the 'grapevine' play for much of non-Soviet research. However, although the editor states that he has tried to include "those fields in which Soviet physics is pursuing a direction of its own" it is not clear in most cases that a review covering non-Soviet (or all) work in the area would necessarily be very different in style or emphasis — which perhaps goes to show that the communication gap is not all that serious after all!

The translation is mostly of a high standard, but I hope that in future volumes the series editors will allow more time for proof-reading and also do something about the type-face used; in the present volume the symbols for italic vee and Greek nu are almost indistinguishable, which leads on occasion (e.g. p.261) to extreme confusion. □

A. J. Leggett is Professor of Theoretical Physics at the University of Sussex, Brighton, U.K.